The role of geographic origin and life history stages in invasion ecology - a comparative assessment of Northern European, Ponto-Caspian and North American species

Dissertation

in fulfillment of the requirements of the degree “Dr. rer. nat.”

of the Faculty of Mathematics and Natural Sciences at Kiel University

Submitted by

Filipa Paiva

Kiel, 2020

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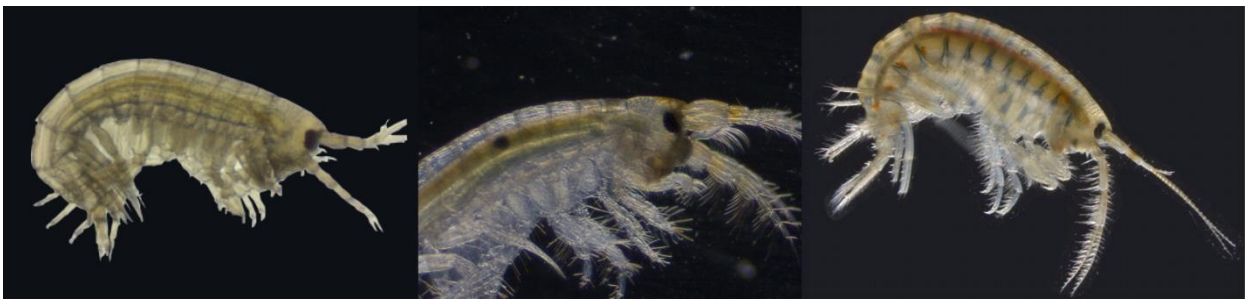

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Cover Illustration - From left to right: *Gammarus salinus* (native to Northern Europe); *Pontogammarus maeoticus* (native to the Ponto-Caspian region); and *Gammarus tigrinus* (native to North America).

First referee: PD Dr. Elizabeta Briski

Second referee: Prof. Dr. Martin Wahl

Date of oral examination: 14.12.2020

Valeu a pena? Tudo vale a pena

Se a alma não é pequena.

Fernando Pessoa – Mar Português

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Summary

The biological composition of most of the earth's major ecosystems is being dramatically changed by human activities. The breakdown of natural barriers, as a consequence of an increasingly connected world, has contributed to a rise in biological invasions worldwide with thousands of non-indigenous species established in freshwater, brackish, and marine ecosystems. Identifying traits correlated with invasion success is a central goal in invasion ecology to predict and prevent future invasions. This dissertation is divided into five chapters. Chapter 1 gives a general introduction to the main topic of the thesis, including invasion ecology and possible determinant factors that might influence invasion success such as geographic origin and life history stages. Furthermore, it also explores the influence of experimental design on results in ecology. In Chapter 2, I question the role of geographic origin on invasion success, specifically, whether Ponto-Caspian species are better able to acclimatize to and colonize habitats across a range of salinities than taxa from Northern European and North American regions. The experiments, using eight gammarid species native to those three regions, demonstrated that although species from all three tested regions indicated high tolerance to a wide range of salinities, significant differences in the direction of salinity tolerance were observed among the regions, with Northern European species having a better survival in higher salinities, and Ponto-Caspian species in lower salinities. Therefore, it is important to consider geographic origin as a predictor of invasion success because it might foresee pre-adaptation of certain species due to its evolutionary history. Following these findings, in Chapter 3, I further compare the salinity tolerance of adults and juveniles of three gammarid species originating from Northern European, the Ponto-Caspian and North American regions to determine whether juveniles tolerate salinity changes equally well as adults. During the invasion process, individuals must overcome several challenges and be able to survive and

reproduce to establish a successful population. Thus, the role of life history stages in the context of invasion ecology is important to consider. While experimental results determined that both adults and juveniles of all three species endured wide ranges of salinity, juveniles tolerated a narrower salinity range than their parents. The evidence from this study emphasizes the importance of testing several life history stages when constructing models to predict future invasions. In Chapter 4, bearing in mind that the approaches used to test scientific questions may differ not only in spatial scale but also in ecological complexity, I explored how the type of experiment (i.e., scale and ecological complexity) affects the outcome and to what extent the two types of experiments are comparable. Two experiments differing in size and ecological-complexity (i.e. outdoor large-scale community-level mesocosm vs. indoor small-scale two-species laboratory experiment), were conducted to assess the effects of marine heatwaves on two gammarid species. The results revealed that while for one species the population growth was similar independently of the size and ecological-complexity, for the other species, the inclusion of the community seemed to have benefited the species' growth rate, demonstrating stronger performance in the mesocosm than in the laboratory experiment. These results suggest the importance of biotic interactions and complexity of natural environments in buffering or boosting the effects of environmental stress on organisms while carrying out ecological experiments. Finally, Chapter 5 summarizes the findings from all experiments and concludes that not only geographic origin and life history stages need to be considered in invasion ecology, but also the approach when selecting our experimental designs to answer research questions.

Zusammenfassung

Die biologische Zusammensetzung der meisten großen Ökosysteme der Erde wird durch menschliche Aktivitäten dramatisch verändert. Die Auflösung natürlicher Barrieren als Folge einer zunehmend vernetzten Welt hat zu einem weltweiten Anstieg biologischer Invasionen beigetragen mit Tausenden nicht heimischen Arten, die sich in Süßwasser-, Brack- und Meeresökosystemen etabliert haben Die Identifizierung von Merkmalen, die mit dem Invasionserfolg korrelieren, ist ein zentrales Ziel der Invasionsökologie um künftige Invasionen vorherzusagen und zu verhindern. Die vorliegende Dissertation ist in fünf Kapitel gegliedert. Kapitel 1 ist eine allgemeine Einführung und behandelt die Invasionsökologie und mögliche den Invasionserfolg beeinflussende Faktoren, wie z.B. die geographische Herkunft und Entwicklungsstadien der eingeführten Arten. In Kapitel 2 habe ich die Rolle der geographischen Herkunft für den Invasionserfolg von Flohkrebsarten untersucht. Insbesondere, ob Ponto-Kaspische Arten besser in der Lage sind, sich an aquatische Lebensräume verschiedener Salzgehalte zu akklimatisieren und diese zu besiedeln, als Arten aus Nordeuropa und Nordamerika. Obwohl alle Arten aus den drei getesteten Regionen ein breites Salzgehaltsspektrum tolerieren, zeigten die Experimente mit acht Gammarid-Arten, dass es zwischen den Arten aus den verschiedenen Regionen signifikante Unterschiede in der Richtung der Salzgehalts-Toleranz gab. Dabei hatten nordeuropäische Arten in höheren und Ponto-Kaspische Arten in niedrigeren Salzgehalten höhere Überlebensraten. Die Evolutionsgeschichte von Herkunftsregionen kann zu Voradaptionen von Arten führen, weshalb die geographische Herkunft zur Vorhersage von Invasionserfolg herangezogen werden könnte. Im Anschluss an diese Ergebnisse habe ich in Kapitel 3 die Salinitätstoleranz von adulten und juvenilen Tieren von drei Gammariden-Arten aus Nordeuropa, der Ponto-Kaspischen und nordamerikanischen Region untersucht. Während des Invasionsprozesses müssen die Individuen

mehrere Herausforderungen überwinden und in der Lage sein, zu überleben und sich fortzupflanzen, um eine erfolgreiche Population zu gründen. Daher ist es wichtig, die Rolle der Entwicklungsstadien im invasionsökologischen Kontext zu verstehen. Die Ergebnisse der Experimente zeigten, dass sowohl adulte als auch juvenile Tiere aller drei Arten weite Salzgehaltsbereiche tolerierten. Dabei tolerierten die Jungtiere einen engeren Salzgehaltsbereich als die Elterntiere. Es ist daher wichtig, mehrere Entwicklungsstadien zu testen um Vorhersagemodelle für zukünftige Invasionen zu erstellen. In Kapitel 4 habe ich untersucht, ob der Umfang und die ökologische Komplexität eines Experiments einen Einfluss auf die Ergebnisse haben. Zwei Experimente, die sich in Größe und ökologische Komplexität unterscheiden, (d.h. Mesokosmen mit einer vielfältigen Artzusammensetzung im Vergleich zu Laborexperimenten mit zwei Arten) wurden durchgeführt, um die Auswirkungen mariner Hitzewellen auf zwei Gammaridenarten zu bewerten. Die Ergebnisse zeigen, dass bei einer Art das Populationswachstum ähnlich war, unabhängig von Größe und ökologischer Komplexität des Experiments, während die Wachstumsrate der anderen Art durch die Einbeziehung der Gemeinschaft in den Mesokosmen begünstigt worden zu sein schien. Diese Ergebnisse verdeutlichen, dass biotische Interaktionen und eine komplexe natürliche Umwelt die Auswirkungen von Umweltstress auf Organismen beeinflussen können. Dies sollte bei der Durchführung ökologischer Experimente beachtet werden. Das fünfte Kapitel fasst die Ergebnisse von allen Experimenten zusammen und zeigt abschließend, dass nicht nur die geographische Herkunft und die Entwicklungsstadien in Invasionsökologie berücksichtigt werden sollten, sondern auch die Wahl des experimentellen Designs wichtig ist um ökologische Forschungsfragen zu beantworten.

Chapter 1

General Introduction

Global change

The cumulative impacts of humans have been affecting terrestrial and aquatic ecosystems worldwide in the past decades (Halpern et al., 2008). Climate change, habitat destruction, eutrophication, pollution and the introduction of non-indigenous species (NIS) represent, among others, ongoing global stressors which, individually or in combination, can lead to profound alterations in community structure, ecosystem functioning and provision of ecosystem services (Capinha et al., 2015; Chapman, 2016). In addition, extreme isolated events associated with global warming (i.e., heatwaves) have been reported more often worldwide (e.g., Garrabou et al., 2009; Pearce & Feng, 2013), with devastating consequences for both marine and freshwater environments (e.g., Hughes et al., 2017; Joehnk et al., 2008). Overall, considering that such environmental disturbance is expected to increase in severity and frequency, it is paramount to understand the responses and resilience of ecosystems to be able to protect and mitigate the negative impacts on coastal habitats (Frölicher et al., 2018; Sorte et al., 2010).

Invasion ecology

The introduction of novel species to a region often results in biological invasions. Biological invasions have gained attention in the early 1990s due to rising economic and ecological issues related to NIS (Elton, 2020). Because of the complex and often long-term direct and indirect impacts on invaded environments, invasions started to be considered as a tool for basic research

used to study the ecology and evolution of populations, and as a conservation subject tied to the protection of biodiversity (Pyšek et al., 2020). The movement of species translocated from one region to another has reached unprecedented numbers, these being higher than any previously observed as a result of globalization and rapid increases in trade and travel (Capinha et al., 2015; Kaluza et al., 2010). Consequently, the effects are felt in the distribution and abundance of native species and food chains worldwide (Hulme, 2009; Ruiz et al., 2000a). Both freshwater and marine ecosystems are greatly affected by NIS, only 16% of all marine ecoregions worldwide existing without the presence of an NIS (Molnar et al., 2008; Spalding et al., 2007). Shipping, mainly through hull fouling and ballast water, and commercial aquaculture have been the major pathways for NIS introductions globally, primarily affecting the temperate regions of Europe, North America, Asia and Australia (Bax et al., 2001; Carlton & Geller, 1993; Casties et al., 2016; Molnar et al., 2008; Seebens et al., 2016).

The process of invasion is far from being simple and species have to pass through a series of stages before becoming invasive. Only some individuals will survive from one stage to another (Lockwood et al., 2013). A simplistic model was created to facilitate the understanding of the invasion process, which comprises four major stages: Transport (when a species is moved outside its native range to a new habitat); Introduction (when the transported organisms are released into the new environment), Establishment (when an NIS establishes a viable population) and Spread (when the established population increases its abundance and expands its geographic range; Fig. 1; Blackburn et al., 2011; Colautti & MacIsaac, 2004; Kolar and Lodge, 2001; Lockwood et al., 2013). Consequently, at least a subset of a population has to be entrained into a transport vector, survive transport and cope with the environment of the new habitat to become established (Blackburn et al., 2009; Simberloff, 2009). Nevertheless, even when individuals of an introduced

population are able to survive and reproduce, if the population growth rate is negative, they can still fail to establish, which particularly may be the case when small populations are introduced (Blackburn et al., 2011).

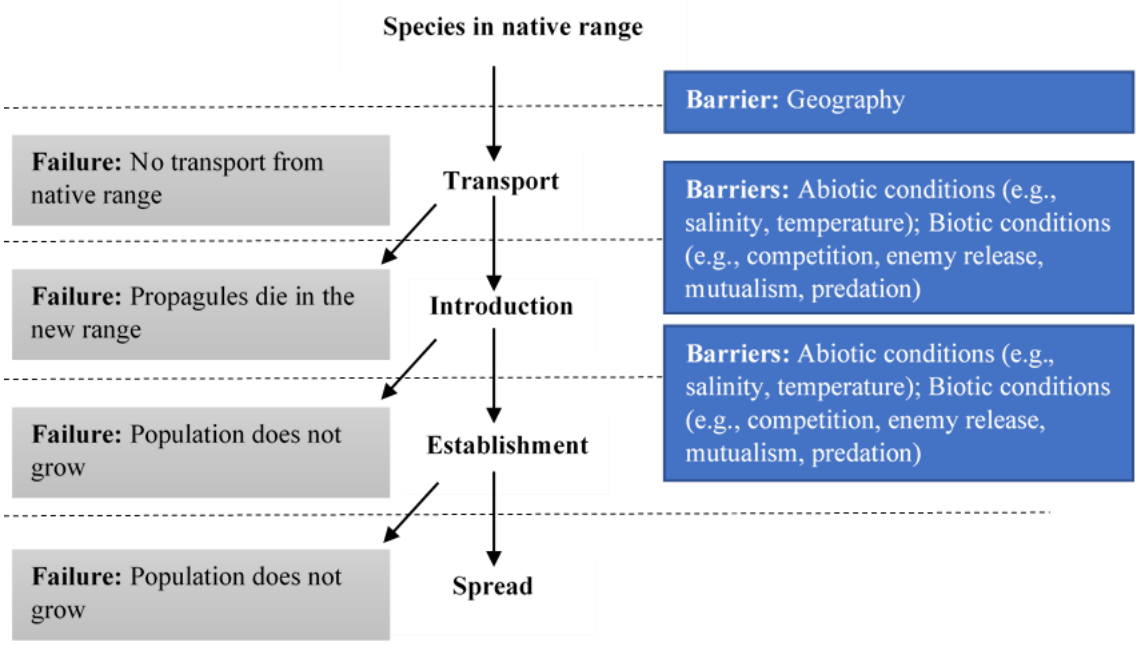

Fig. 1. Invasion process model modified from Lockwood et al. (2013) and Darrigran & Damborenea (2015).

One of the fundamental goals in invasion ecology science is the identification of factors that increase the risk of establishment success of NIS. Propagule pressure, which is defined as the quantity, quality and/or frequency of introduced individuals, seems to play a crucial role in the invasion process, as propagules arriving from a donor region influence establishment probability (Lockwood et al., 2005; Simberloff, 2009). Characteristics of individuals within the introduced population, such as phenotypic plasticity and preadaptation to variable environments, can also contribute to a higher survival rate during the invasion process. These attributes maintain a high propagule pressure, consequently leading to a successful invasion (Blackburn et al., 2009; Hercus, 2000; Hoffmann & Lande, 2015; Simberloff, 2009). Likewise, selection during the transport stage

of the invasion process may also facilitate local adaptation (e.g., survival of only pre-adapted individuals for particular environmental conditions; Briski et al., 2018). Finally, an overlooked aspect influencing the success of invasive species seems to be the geographic origin of the species despite assumptions of several previous studies highlighting that certain geographic regions are major donors of NIS, in particular, those with disturbed geological history and environmental fluctuations (Bij de Vaate et al., 2002; Casties et al., 2016; Reid & Orlova, 2002; Ricciardi & MacIsaac, 2000).

The Ponto-Caspian, Northern European and North American Regions

The opening of canals connecting rivers and global shipping traffic, contributed to the dispersal of aquatic organisms, supplying these with many opportunities for further distribution (Casties et al., 2016; Galil et al., 2008). The Ponto-Caspian region (Black Sea, Sea of Azov and Caspian Sea) represents one of the major donors of NIS to both the Great Lakes in North America, and Northern European regions (Casties et al., 2016). However, while the numbers of Ponto-Caspian species are increasing in both, only a small number of species from those regions are found in the Ponto-Caspian region (Reid & Orlova, 2002; Leppäkoski et al., 2010). In addition, there is also a small number of species found between the Great Lakes - St. Lawrence River region and Northern European region, albeit ship transport routes between the two regions is relatively high and of similar intensity in both directions (Kaluza et al., 2010). As proposed by previous studies, the possible reason behind this asymmetrical transfer of species among regions might be related to the origin of Ponto-Caspian species and the region's geological history (Casties et al., 2016; Leppäkoski et al., 2002; Reid & Orlova, 2002; Ricciardi & MacIsaac, 2000). In the evolution of the Ponto-Caspian basins is reflected the extensive environmental fluctuations that affected the region for millions of years (i.e., from 2.5 million years ago to 10,000 years ago), from fully marine

environments, as part of the Tethys Sea, to almost pure freshwater ecosystems such as the Sarmatian Sea (Reid & Orlova, 2002; Zenkevitch, 1963). During the formation of the seas that we now know as Black, Azov and Caspian Seas, several connections and disconnections were established, with the Mediterranean Sea causing additional changes in salinity until, finally, the system stabilized with salinity ranging from complete freshwater to marine (Fig. 2; Reid & Orlova, 2002; Zenkevitch, 1963). As a result, these events had a strong influence on the fauna of the Ponto-Caspian region, in particular, the endemic species which evolved from relict immigrants and were selected for their euryhalinity (Reid & Orlova, 2002).

The complex climatic changes of the Ponto-Caspian region contrast greatly with the evolutionary histories of Northern Europe and the Great Lakes in North America. The ecosystems of the Baltic Sea and the Great Lakes were formed after the last glacial period and are less than 12 000 years old (Leppäkoski et al., 2002; Reid & Orlova, 2002). The Baltic Sea is a relatively isolated water body, which is connected to the Atlantic Ocean through the narrow passage to the North Sea (HELCOM, 2018; Reid & Orlova, 2002). Through this connection, species found in the Baltic Sea are a mixture of migrants from the Atlantic Ocean and some Arctic glacial relicts (Reid & Orlova, 2002). After the last glacial period, the Baltic Sea went through several changes in salinity, from freshwater to marine, and currently, it is characterized by a brackish water gradient where salinity can range from 15-18 ppt at the entrance, to 0-2 ppt in the northeast part (Fig. 2; HELCOM, 2018). As young as the Baltic Sea, the Great Lakes became the largest freshwater reservoir on earth after the retraction and meltdown of the last glaciers, and are connected to the Atlantic Ocean through the St. Lawrence River (Reid & Orlova, 2002). In the modern lakes, ancient species from the brackish Arctic Ocean dominate the benthic community in conjunction with a relatively high endemic species diversity that can be found among several faunal groups (Reid & Orlova, 2002).

Geological evidence suggests that neither saltwater nor brackish water were ever present in the basins, which suggests that the Great Lakes are exclusively freshwater (Fig. 2; Reid & Orlova, 2002).

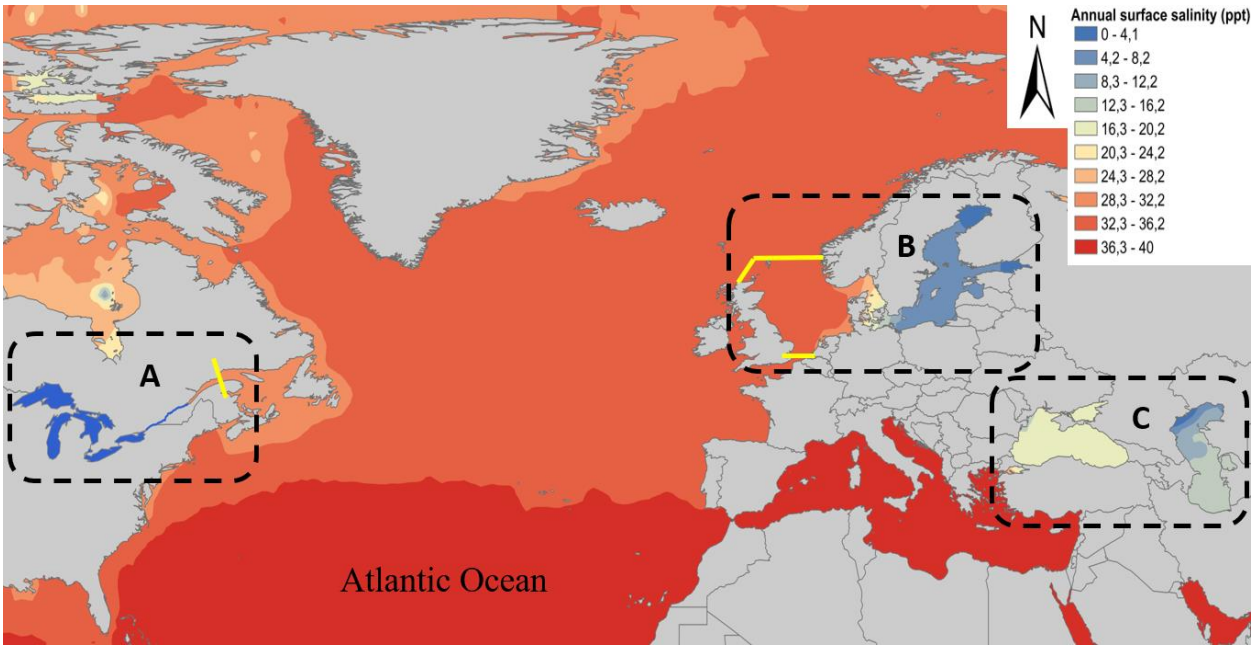

Fig. 2. Geographic areas studied: **A** - Great Lakes - St. Lawrence River Region; **B** - Northern European Region; and **C** - Ponto-Caspian Region. Map constructed using average annual salinity data with a 1° x 1° spatial resolution from the World Ocean Atlas database (Antonov et al., 2006). The salinity of the Great Lakes is below 0.5 ppt (i.e., freshwater). Yellow lines delimit regions (modified from Casties et al. 2016).

Throughout history, the geographic isolation between plants and animals among these three regions has been gradually destroyed by the deliberate or accidental transport of NIS. Successful colonization of Ponto-Caspian species in both brackish and freshwater habitats of Northern Europe and the Great Lakes, respectively, has led to consider the possibility that Ponto-Caspian species are, in fact, of freshwater origin rather than marine origin, and the disturbed geological history of their native region may have favored their establishment in freshwater habitats (Bij de Vaate et al., 2002; Casties et al., 2016; Mordukhay-Boltovskoy, 1964; Reid & Orlova, 2002). Therefore, an increasing number of studies have investigated the potential salinity tolerance of Ponto-Caspian

species to brackish and freshwater habitats, since species from this particular region are more likely to be found in habitats with less salinity than their native range (Dobrzycka-Krahel & Graca, 2018; Kobak et al., 2017; Pauli & Briski, 2018). An alternative explanation, contrary to the above-mentioned suggestions of a freshwater origin of Ponto-Caspian NIS, may rely on the hypothesis that Ponto-Caspian taxa do in fact have a marine origin, but adapted through time to freshwater environments while moving northwards through European freshwater canals. However, while considering this theory, we must ask why the same pattern was not followed by Northern European species over the same time span.

Salinity and life history stages

Salinity was assumed to be an important limiting factor for most aquatic species that influenced species dispersal from marine to brackish and freshwater habitats, and *vice versa* (Dahl, 1956). Nonetheless, numerous marine and brackish NIS have been frequently and recently reported in freshwater habitats, and only a few freshwater NIS reported as established in brackish environments (Casties et al., 2016; Grigorovich et al., 1998; Lee & Bell, 1999; Ricciardi & MacIsaac 2000; Ruiz et al., 1997; Sylvester et al., 2013). While entering freshwater habitats, marine organisms must evolve to retain osmotic levels in body fluids in order to compensate for salinity alterations. Such compensation requires a high energy cost from the organism, which may not only compromise major physiological needs but also have negative consequences on reproduction, development, growth and survival of the stressed individuals (Anger, 2003; Łapucki & Normant, 2008; Morgan & Iwama, 1999; Neuparth et al., 2002; Normant & Lamprecht, 2006). During the invasion process, individuals must survive through the four major stages to establish a successful population. Later, in the course of invasion, the physiological quality of the individuals transported reduces and even though adult organisms can tolerate a wide range of salinities, they

still may not be able to reproduce (Steele & Steele, 1991 and references therein). Although, if reproduction is successful, it is still possible that their offspring will be affected by the parents' exposure to stress. This can result in poor embryo viability, a decreased number of broods and a reduced number of emergent juveniles (e.g., Mills & Fish, 1980, Steele & Steele, 1991; Vlasblom & Bolier, 1971).

Aquatic enclosed experiments

Research in aquatic habitats is challenging because interactions can occur over a broad time-scale, space and ecological complexity, which difficult controlled research (Widdicombe et al., 2010). Field observations of natural fluctuations and experimental approaches, often in combination with modeling, are frequently performed to understand coastal ecosystems and their resilience to stress (Petersen et al., 2009; Widdicombe et al., 2010). Aquatic enclosed experiments, such as large-scale mesocosms and small-scale laboratory experiments, have gained in popularity as research tools in aquatic ecosystems, partially because they provide scientists valuable information with a certain degree of control of environmental factors, that otherwise would not be possible through field experiments. Through the course of time, due to scientific and technological developments, new and more advanced approaches and methods have been designed, including valuable progress in experimental set-ups, data analyses and computation (Evans, 2012; Woodward et al., 2010).

Laboratory experiments are frequently used to test basic biological, chemical and physical questions in a controlled environment and are conducted at small-scale and individual or single-species levels, allowing for highly replicated experimentation under controlled conditions (Widdicombe et al., 2010). While creating an artificial environment, the accuracy of such experiments is doubted when important ecological and biological components present under

natural settings are excluded, which often raises concern among the scientific community (Carpenter, 1996; Stewart et al., 2013; Widdicombe et al., 2010). In contrast, large-scale mesocosm experiments have, in recent years, gained recognition by offering the possibility of experimenting in a more realistic context using subsets of natural ecosystems while allowing replication (Pansch & Hiebenthal, 2019; Wahl et al., 2015). Nevertheless, while the resemblance to natural ecosystems is more evident here than in laboratory experiments, mesocosm experiments still keep species in an enclosed environment and, therefore, it is still a simplification of what occurs in nature (Petersen et al., 2009; Widdicombe et al., 2010). Both types of aquatic enclosed experiments (i.e., large-scale mesocosm and small-scale laboratory experiments) represent a potentially powerful tool for testing and expanding our understanding of the mechanisms that influence ecological dynamics. However, approaches comparing and extrapolating data that might differ in terms of space and ecological complexity remain poorly understood.

Thesis objectives

This doctoral thesis describes the role of geographic origin and life history stages in invasion ecology considering the Ponto-Caspian, the Northern European and the North American regions. In Chapter 2, I investigated if Ponto-Caspian taxa more readily acclimatize to and colonize diverse salinity habitats than taxa from the Northern European and Great Lakes - St. Lawrence River regions. For that, laboratory experiments were performed on 22 populations of eight gammarid species native to those three regions. In addition, I conducted a literature search to survey salinity ranges of tested species worldwide. Finally, possible evolutionary relationships were explored among the examined species and the different populations of the same species.

In Chapter 3, I explored whether juveniles demonstrated the same salinity tolerance as their parents of one Northern European (*Gammarus salinus*), one Ponto-Caspian (*Pontogammarus maeoticus*) and one North American species (*Gammarus tigrinus*). Additionally, I compared the results of these experiments with those in Chapter 2, since the salinity tolerance was also tested in adults.

In Chapter 4, I conducted two types of experiments run at different scale and ecological complexity levels (i.e., outdoor large-scale community-level mesocosm vs. indoor small-scale two-species laboratory experiment) to assess the effects of marine heatwaves on two gammarid species from the Baltic Sea.

Chapter 2

Is salinity an obstacle for biological invasions?

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Filipa Paiva [1,2], Andrea Barco[1], Yiyong Chen[3,4], Alireza Mirzajani[5], Farrah T. Chan[6], Velda Lauringson[7], Miguel Baltazar-Soares[1,8], Aibin Zhan[3,4], Sarah A. Bailey[6], Jamileh Javidpour[1,] and Elizabeta Briski[1]

[1]GEOMAR, Helmholtz-Zentrum für Ozeanforschung Kiel, Düsternbrooker Weg 20, 24105 Kiel, Germany

[2]MARE – Marine and Environmental Sciences Centre, Quinta do Lorde Marina, Sítio da Piedade, 9200-044, Caniçal, Madeira Island, Portugal

[3]Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, 18 Shuangqing Road, Haidian District, Beijing 100085, China

[4]University of Chinese Academy of Sciences, 19A Yuquan Road, Shijingshan District, Beijing 100049, China

[5]Inland Water Aquaculture Research, Taleghani Street NN, Bandar Anzali, Iran

[6]Great Lakes Laboratory for Fisheries and Aquatic Sciences, Fisheries and Oceans Canada, 867 Lakeshore Road, Burlington, Ontario, L7S 1A1, Canada

[7]Estonian Marine Institute, University of Tartu, Vanemuise 46, 51014 Tartu, Estonia

[8]Faculty of Science and Technology, Bournemouth University, Christchurch House, Talbot Campus, Poole, Dorset BH12 5BB, United Kingdom of Great Britain and Northern Ireland

Abstract

Invasions of freshwater habitats by marine and brackish species have become more frequent in recent years with many of those species originating from the Ponto-Caspian region. Populations of Ponto-Caspian species have successfully established in the North and Baltic Seas and their adjoining rivers, as well as in the Great Lakes–St. Lawrence River region. To determine if Ponto-Caspian taxa more readily acclimatize to and colonize diverse salinity habitats than taxa from other regions, we conducted laboratory experiments on 22 populations of eight gammarid species native to the Ponto-Caspian, Northern European and Great Lakes–St. Lawrence River regions. In addition, we conducted a literature search to survey salinity ranges of these species worldwide. Finally, to explore evolutionary relationships among examined species and their populations, we sequenced the mitochondrial cytochrome *c* oxidase subunit I gene (COI) from individuals used for our experiments. Our study revealed that all tested populations tolerate wide ranges of salinity, however, different patterns arose among species from different regions. Ponto-Caspian taxa showed lower mortality in fresh water, while Northern European taxa showed lower mortality in fully marine conditions. Genetic analyses showed evolutionary divergence among species from different regions. Due to the geological history of the two regions, as well as high tolerance of Ponto-Caspian species to fresh water, whereas Northern European species are more tolerant of fully marine conditions, we suggest that species originating from the Ponto-Caspian and Northern European regions may be adapted to freshwater and marine environments, respectively. Consequently, the perception that Ponto-Caspian species are more successful colonizers might be biased by the fact that areas with highest introduction frequency of NIS (i.e., shipping ports) are environmentally variable habitats which often include freshwater conditions that cannot be tolerated by euryhaline taxa of marine origin.

Keywords: Freshwater origin, Gammaroidea, marine origin, non-indigenous species, Ponto-Caspian species, salinity tolerance

Introduction

The anthropogenic movement of species is one of the leading threats to biodiversity resulting from globalization and rapid increases in trade and travel (Capinha, Essl, Seebens, Moser, & Pereira, 2025; Hulme, 2009; Kaluza, Kölzsch, Gastner, & Blasius, 2010; Ruiz, Fofonoff, Carlton, Wonham, & Hines, 2000). To date, thousands of non-indigenous species (NIS) have established in freshwater, brackish, and marine ecosystems facilitated by anthropogenic vectors, mainly shipping, aquaculture, and canal construction (Molnar, Gamboa, Revenga, & Spalding, 2008; Ricciardi, 2006; Ruiz, Rawlings, et al., 2000; Vitousek, D'Antonio, Loope, Rejmanek, & Westbrooks, 1997). To become established, at least a subset of a population has to be entrained into a transport vector, to survive transport, as well as the environment of the subsequent new habitat (Blackburn, Lockwood, & Cassey, 2009; Simberloff, 2009). Empirical and statistical evidence support propagule pressure, which is defined as the quantity, quality, and/or frequency of introduced individuals (Lockwood, Cassey, & Blackburn, 2005; Simberloff, 2009), as a main factor determining invasion success (Blackburn et al., 2009; Mack et al., 2000; Simberloff, 2009; Williamson & Fitter, 1996). Higher propagule pressure yields higher probability of success, yet many introduced populations seem to fail to establish in new habitats (Blackburn et al., 2011). Characteristics of individuals within the introduced population, such as phenotypic plasticity and preadaptation to variable environments, may facilitate survival during the invasion process, maintaining high propagule pressure leading to invasion success (Blackburn et al., 2009; Hoffmann & Hercus, 2000; Lande, 2015; Simberloff, 2009).

Salinity is considered one of the most significant factors limiting the distribution of species in aquatic environments (Grabowski, Bacela, & Konopacka, 2007; Ojaveer et al., 2010), however, the establishment of marine and brackish NIS in freshwater habitats has been frequently reported in recent years, with many of those species originating from the Ponto-Caspian region (i.e., Black, Azov, and Caspian Seas; Ruiz, Carlton, Grosholz, & Hines, 1997; Lee & Bell, 1999; Ricciardi & MacIsaac, 2000; Casties, Seebens, & Briski, 2016). On the contrary, only few freshwater NIS have been reported as established in brackish, but not marine, environments (Grigorovich, Pashkova, Gromova, & van Overdijk, 1998; Sylvester, Cataldo, Notaro, & Boltovskoy, 2013). There are 17 freshwater phyla, all of which originated from marine environments in evolutionary history (Briggs, 1995; Lee & Bell, 1999; Little, 1990). While marine organisms entering freshwater habitats must evolve to retain osmotic levels in body fluids, which requires high energetic costs, freshwater taxa entering marine environments must evolve to maintain lower body fluid concentrations relative to the highly concentrated environment (Łapucki & Normant, 2008; Morgan & Iwama, 1999; Schubart & Diesel, 1999). Consequently, one would assume that brackish or freshwater species would more readily invade marine environments than vice versa; but invasions of Northern Europe and the Laurentian Great Lakes by Ponto-Caspian species show the opposite pattern (Casties et al., 2016; Ricciardi & MacIsaac, 2000).

Ponto-Caspian species have established nonindigenous populations in both brackish and freshwater habitats of the North and Baltic Seas and their adjoining rivers, as well as in the Great Lakes–St. Lawrence River region (Bij de Vaate, Jażdżewski, Ketelaars, Gollasch, & Van der Velde, 2002; Casties et al., 2016; Reid & Orlova, 2002; Ricciardi & MacIsaac, 2000). Previous studies suggested that marine Ponto-Caspian taxa established in freshwater habitats might be of freshwater origin due to the geological history of the Ponto-Caspian region (Casties et al., 2016; Reid & Orlova, 2002). Ten million years ago during the Miocene, after the separation of the

Sarmatian Lake from the Tethyan Ocean, the salinity of the enclosed lake started to decrease, being several times almost completely dry during Glacial Maxima (i.e., from 2.5 million years ago to 10,000 years ago) with freshwater flooding after ice melting at the end of each Glacial Maximum. During that period there were also few geological connections and disconnections of the region with the Mediterranean Sea causing several additional changes in salinity until finally the system was shaped as the Black, Azov, and Caspian Seas, with salinity ranging from freshwater to marine (i.e., 30 g/kg; Reid & Orlova, 2002; Zenkevitch, 1963). In contrast, taking into account almost two centuries of spread of Ponto-Caspian species through European freshwater canals, an alternative explanation may be that Ponto-Caspian taxa have marine origin but adapted to freshwater environments while moving northward.

To understand the invasion pattern, a nearly unidirectional invasion of Ponto-Caspian species to other regions but not vice versa, we explored if Ponto-Caspian taxa are better able to acclimatize to and colonize habitats across a range of salinities than are taxa from other regions. We conducted laboratory experiments on 22 populations of eight gammarid species native to the Ponto-Caspian, Northern European, and Great Lakes–St. Lawrence River regions. The superfamily Gammaroidea was chosen due to its history of colonizing numerous geographic regions with different salinity patterns (Rewicz, Wattier, Grabowski, Rigaud, & Bącela-Spychalska, 2015; Szaniawska, Lapucki, & Normant, 2003). We tested the hypotheses that there is no difference in salinity tolerance among: (i) different populations of the same species; (ii) different species from the same region; and (iii) species from different regions. In addition, we conducted a literature search using Thomson's Institute for Science Information (ISI) Web of Knowledge to determine reported salinity ranges of indigenous and nonindigenous areas for each species. Finally, to explore evolutionary relationships among examined species and their

populations, we sequenced the mitochondrial cytochrome *c* oxidase subunit I gene (COI) from individuals in all populations used for our experiments.

Materials and methods

Laboratory experiments

The salinity experiments were conducted from May to November 2015 and from April to June 2016. Eight species were tested: *Gammarus locusta, G. oceanicus, G. salinus,* and *G. zaddachi*—native to Northern Europe; *Pontogammarus maeoticus* and *Obesogammarus crassus*—native to the Black and Caspian Seas; *G. tigrinus*—native to eastern North America; and *G. fasciatus*—native to the Laurentian Great Lakes. At least two populations, preferably originating from different salinities, were tested for each species, except for *G. oceanicus* (one population tested). Populations of seven species were collected and tested in their native areas, whereas those of *G. tigrinus*, due to practicality and distance from available testing stations, were collected and tested in invaded regions (Appendix S1). All populations except *G. fasciatus* were collected from areas with annual salinity variations of a few g/kg; however, none of the collection sites have freshwater conditions except *G. fasciatus*.

After collection, individuals were transported in ambient water to the laboratories where each individual was morphologically identified according to Köhn and Gosselck (1989) for *G. locusta, G. salinus, G. zaddachi, and G. oceanicus*; Holsinger (1972) for *G. fasciatus*; Lincoln (1979) for *G. tigrinus*; and Birstein and Romanova (1968), Moiceiev and Filatova (1985), Sars (1896), Stock (1974), and Stock, Mirzajani, Vonk, Naderi, and Kiabi (1998) for *P. maeoticus* and *O. crassus*. Morphological identification was confirmed by randomly separating up to 30 morphologically identified individuals for DNA barcoding using COI in the case of seven species, while *G. fasciatus* was confirmed by 16S rDNA (protocol provided below).

Following 2 weeks of acclimatization to laboratory conditions at ambient salinity and temperature (same as the collection site), salinity tests were conducted using 10 mean-size adult individuals per replicate for tests with *G. locusta, G. salinus, G. zaddachi, G. oceanicus, G. tigrinus,* and *G. fasciatus*, while 30 individuals per replicate were used for *P. maeoticus* and *O. crassus*. Given that adults do not exhibit a distinct sexual dimorphism, except when females are ovigerous, sex was not specified. The experimental design consisted of three treatments: (i) control (except for *P. maeoticus* and *O. crassus*); (ii) increased salinity; and (iii) decreased salinity. Each treatment in the experiments was tested in triplicate. The water used for experiments was natural water collected locally near each institute, and filtered through a 20 μm filter. The salinity of the control treatments was identical to the ambient water of collection site. The increased and decreased salinity treatments began at the ambient salinity of the population collection site, which was then increased/decreased by 2 g/kg every 2 days, respectively (Delgado, Guerao, & Ribera, 2011). Increased salinity was achieved by adding artificial seawater (Instant Ocean®) to the local filtered water for all populations except for *P. maeoticus* and *O. crassus*; in the case of *P. maeoticus* and *O. crassus*, salinity was increased by evaporating the local filtered water. Decreased salinity was achieved by adding potable tap water to dilute the local filtered water. Mortality was checked daily throughout the experiments. When the values of 0 and 40 g/kg were reached, mortality was followed up for two more weeks, as well as the presence of offspring. As sex was not determined, we acknowledge that the ratio of male/female adults in each replicate could be inconsistent, although highly unlikely that all individuals in a replicate were of the same sex, leading to no offspring observation for the replicate. The primary dataset containing experimental results is available at: https://doi.org/10.1594/pangaea.884715.

Statistical Analysis of Salinity Experiments

We tested for differences in the onset and rate of mortality between populations within species and between treatments within populations. To test for differences within species and within populations, we constructed a mortality curve for each treatment for each population, and for each population for each species, using pooled data from all replicates, described by the equation (Briski, Ghabooli, Bailey, & MacIsaac, 2011; Briski, VanStappen, Bossier, & Sorgeloos, 2008):

$$y=100/[1+e\text{-}Z(s\text{-}Q)] \quad (1)$$

where s is salinity change (i.e., change in g/kg), Z is the rate of mortality, and Q is the onset of mortality (i.e., percentage mortality). The model was expanded to compare the rate and the onset of mortality between two curves using the equation (Briski et al., 2008, 2011):

$$y=100/[1+e\text{-}(Z_1+Z_2)(s\text{-}Q_1\text{-}Q_2)] \quad (2)$$

where Z_1 and Z_2 are the rates of mortality, and Q_1 and Q_2 are the points of onset of mortality, for the first and second curves, respectively. All possible combinations of curve pairs were compared statistically by the Fit Nonlinear Model using Generalized Least Squares. Significance levels for statistical comparisons of estimated parameters Z_1 and Z_2, and Q_1 and Q_2**,** were adjusted for multiple pairwise comparisons by Bonferroni-type correction to guard against inflating the Type I error rate. The family-wise error rate of 0.05 was used. All tests were performed using S-Plus 6.1 (S-Plus® 6.1, 2002, Insightful Corp., Seattle, Wa, USA). Due to high variability across populations, our model did not allow comparisons among species.

Molecular identification, GenBank sequences and data analysis

Total genomic DNA was extracted from the telson of each individual with the innuPREP DNA kit (analitikjena, Jena, Germany) or Marine Animal DNA Kit (TIANGEN, Beijing, China) following manufacturers' instructions. A fragment of the COI gene was amplified using a few different pairs of primers: LCO1490 and HCO2198 (Folmer, Black, Hoeh, Lutz, & Vrijenhoek, 1994), UCOIF and UCOIR (Costa, Henzler, Lunt, Whiteley, & Rock, 2009), and *G. tigrinus* species-specific primers (Kelly, MacIsaac, & Heath, 2006). In the case of *G. fasciatus*, 16S primers and protocols following Zhan, Bailey, Heath, and MacIsaac (2014) were applied. PCR amplifications were carried out in 20 μl volume including 10× DreamTaq Buffer (containing $MgCl_2$), 100 mm dNTPs, 10 mm of each primer, 1–10 ng of genomic DNA, and 1 Unit of DreamTaq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) or 1 Unit of Taq DNA polymerase (Takara China; Dalian, China). The amplification protocol consisted of 5 min denaturation at 94°C, followed by 33 cycles of denaturation at 94°C for 35 s, annealing at 47°C for 45 s, extension at 69°C for 45 s, and a final extension step of 69°C for 10 min. PCR products were cleaned with 0.4 U of FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific) and 4U of Exonuclease I (Thermo Fisher Scientific). Cleaned PCR products were prepared for sequencing using a BigDye Terminator v3.1 cycle sequencing kit (Thermo Fisher Scientific), purified with a BigDye XTerminator Purification Kit (Thermo Fisher Scientific) and sequenced on an automated ABI 3130XL capillary sequencer.

Evolutionary relationships among all species were inferred with phylogenetic trees. Two sequences were added to act as outgroups to the overall dataset (i.e., *Crangonix pseudogracilis*) and within genus (i.e., *Dikerogammarus villosus*; Hou & Sket, 2016). Accession numbers can be found in Appendix S1 (Baltazar-Soares, Paiva, Chen, Zhan, & Briski, 2017). Sequences were

aligned and manually trimmed to a standard fragment size in BioEdit v7.0.4.1 (Hall, 1999). A nucleotide substitution model was estimated using a maximum-likelihood method allowing strong branch swapping. The best-fit model was chosen according to Bayesian inference criteria and was used in the construction of two phylogenetic trees: constructed with the Neighbor-joining method (Saitou & Nei, 1987) implemented in MEGA v6.0 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) and a coalescent-based Bayesian methodology (Kingman, 1982) in BEAST v1.8 (Drummond & Rambaut, 2007). For the Neighbor-joining method, we considered transitions/transversions and statistical support was inferred with 1,000 bootstrap iterations. For the Bayesian method, a molecular clock was set to a strict divergence rate of 2.2% per million years (Cristescu, 2015). All other parameters were retained as defaults. The Neighbor-joining tree was set to "coalescent constant size" and the Markov Chain Monte Carlo length to 1×10^8; convergence was inspected in Tracer v1.6 (Rambaut, Suchard, Xie, & Drummond, 2014). The Bayesian tree chosen for visual representation was selected through Maximum Clade Credibility, considering a posterior probability limit of 0.95 and 1,000 burn-in steps in TreeAnnotator v1.8.0, and drawn in FigTree v1.4.0 (Drummond & Rambaut, 2007).

Salinity range of tested species

To compile the range of salinities of all habitats where the eight species occur in nature, we used Thomson's Institute for Science Information (ISI) Web of Knowledge 5.22.3 to search published data (search conducted between September 2015 and June 2016). The search was performed separately for each species using species name and "topic" as the search field through all available dates. Results were refined by subject area, including topics broadly relevant to ecology, comprising biodiversity conservation, biology, ecology, environmental sciences, freshwater biology, marine biology, oceanography, and zoology. We checked 441 studies

recovered from ISI, and found 151 contained information on the salinity and/or coordinates of an occurrence location (Appendix S2). In cases where only coordinates were provided, salinity was determined for that location using the mean annual salinity from The World Ocean Atlas database (Antonov, Locarnini, Boyer, Mishonov, & Garcia, 2006).

Results

Salinity experiments

In general, all species showed wide ranges of salinity tolerance. However, different patterns arose among species from different regions with those from Northern Europe better tolerating higher salinity and those from the Ponto-Caspian region better tolerating lower salinity (i.e., in the increased salinity and decreased salinity treatments); species from the Great Lakes–St. Lawrence River region did not perform well in laboratory settings—high mortality was observed in the control treatment as well (Table 1 and Appendix S3; Figures 1 and 2). The four Northern European species tested, collected from salinities ranging from 1 to 31 g/kg (Appendix S1), showed no difference in mortality rate among populations of the same species when salinity was increased to 40 g/kg (Table 2; Figure 1). The onset of mortality was different between two populations of *G. locusta* and *G. zaddachi* (i.e., Falckenstein and Helgoland, and Warnemünde and Kronenloch, respectively; Table 2; Figure 1). At 40 g/kg, mortality was lower than 50% and offspring were noted. In the decreased salinity treatment, Northern European species often showed differences among populations either in the onset, mortality rate, or both (Table 2; Figure 2). When salinity reached 0 g/kg mortality was again <50%. However, after 2 weeks at 0 g/kg, mortality increased above 50% for all species except *G. oceanicus* (Figure 2). The presence of offspring was recorded only for two populations of *G. salinus* (i.e., Helgoland and Kiel) but not for the other species.

Table 1 Statistical comparison of parameters between pairs of fitted curves for the increased and decreased treatments, which showed significant difference in the onset, rate of mortality, or both.

Species	Population	Experimental treatment	The onset of mortality (*P*-value)	The rate of mortality (*P*-value)
Gammarus locusta	Helgoland	Increase - Decrease	< **0.0001**	< **0.0001**
	Falckenstein	Increase - Decrease	< **0.0001**	< **0.0001**
	Warnemünde	Increase - Decrease	< **0.0001**	< **0.0001**
Gammarus oceanicus	Kiel	Increase - Decrease	0.4188	0.2585
Gammarus. salinus	Helgoland	Increase - Decrease	0.5789	0.5314
	Falckenstein	Increase - Decrease	**0.0054**	**0.0002**
	Kiel	Increase - Decrease	< **0.0001**	< **0.0001**
	Travemünde	Increase - Decrease	< **0.0001**	0.3022
Gammarus zaddachi	Warnemünde	Increase - Decrease	**0.0002**	**0.0290**
	Kronenloch	Increase - Decrease	< **0.0001**	< **0.0001**
Pontogammarus maeoticus	Jafrud	Increase - Decrease	< **0.0001**	< **0.0001**
	Shafarud	Increase - Decrease	< **0.0001**	< **0.0001**
	Bandar-e Anzali	Increase - Decrease	**0.0037**	**0.0001**
Obesogammarus crassus	Havigh	Increase - Decrease	< **0.0001**	< **0.0001**
	Gisom	Increase - Decrease	< **0.0001**	< **0.0001**
	Chaboksar	Increase - Decrease	< **0.0001**	< **0.0001**
Gammarus tigrinus	Liu	Increase - Decrease	< **0.0001**	**0.0004**
	Pärnu	Increase - Decrease	< **0.0001**	**0.0002**
	Travemünde	Increase - Decrease	0.2191	0.1056

The increased treatment was compared to the decreased treatment of the same population to determine if the population equally tolerates higher and lower salinity stress. The *t*-test incorporated in the Fit Nonlinear Model using Generalized Least Squares was used to test for differences between estimated parameters - rate of mortality (Z_1 and Z_2) and onset of mortality (Q_1 and Q_2). Significant *P*-values are presented in bold. Bonferroni-type protection to guard against inflating the Type I error rate and family-wise error rate of 0.05 were used for pairwise statistical comparisons.

Ponto-Caspian species were collected from salinities ranging from 4 to < 11 ppt (Tables 2 and S1; Figs.1 and 2). In the increased salinity treatment, mortality of all three *P. maeoticus* populations and one *O. crassus* population (i.e., Chaboksar) was 100% at 30 ppt. Two populations of *O. crassus* reached 40 ppt, however, mortality was more than 75% (Table 2; Fig. 1). The onset and mortality rate often differed among *P. maeoticus* populations, while in the case of *O. crassus* there was difference only in the onset of mortality between Havigh population and the two other populations (Table 2; Fig. 1). There were no offspring observed in the increased salinity treatment. In the decreased salinity treatment, there was difference in the mortality rate between Shafarud population and the two other populations of *P. maeoticus*, and the onset of mortality between Havigh and Chaboksar populations of *O. crassus* (Table 2; Fig. 2). Mortality of all populations of both species was less than 30% at the end of the decreased salinity experiment (Fig. 2), with offspring present.

The Great Lakes–St. Lawrence River region species *G. fasciatus* and *G. tigrinus*, collected from salinity < 0.5 ppt and from > 4 to 10 ppt, respectively, revealed high variability in both the onset and mortality rate among populations (Table 2; Figs. 1 and 2). In general, both species did not perform well in laboratory settings, with high mortality in the control treatment (i.e., 94% and 74%, respectively). Mortality of *G. tigrinus* in the increased and decreased treatments was more than 60 % and 50 %, respectively (Figs. 1 and 2), with no offspring observed. Interestingly, *G. fasciatus*, a freshwater species, survived increased salinity treatment up to 40 ppt, though with mortality higher than 60% (Fig. 1); offspring were observed below 27 ppt.

Non-indigenous status and salinity range of tested species

The literature search revealed that all four Northern European species and *G. fasciatus* from the Great Lakes–St. Lawrence River region have not spread outside their native ranges (Table 3).

All species, except *G. fasciatus*, were reported from a very broad range of salinity. While three Northern European species (i.e., *G. locusta, G. oceanicus and G. salinus*) occupy habitats where salinity ranges from 5 to 30 ppt, *G. zaddachi* inhabits both freshwater and marine environments (i.e., from < 0.5 to 30 ppt; Table 3). The Ponto-Caspian species *P. maeoticus* has a broader salinity range in its indigenous (i.e., from 0.5 to 30 ppt) compared to its non-indigenous region (i.e., from 17 to 18 ppt), whereas *O. crassus* inhibits higher salinity in its indigenous (i.e., from 12 to 34 ppt) than in its non-indigenous range (i.e., from < 0.5 to 19 ppt, Table 3). Finally, *G. tigrinus* inhibits both freshwater and marine habitats in both indigenous and non-indigenous regions (i.e., from < 0.5 to 30 ppt, Table 3), with numerous records in freshwater environments in its non-indigenous region. *G. fasciatus* was recorded only from freshwater habitats (< 0.5 ppt).

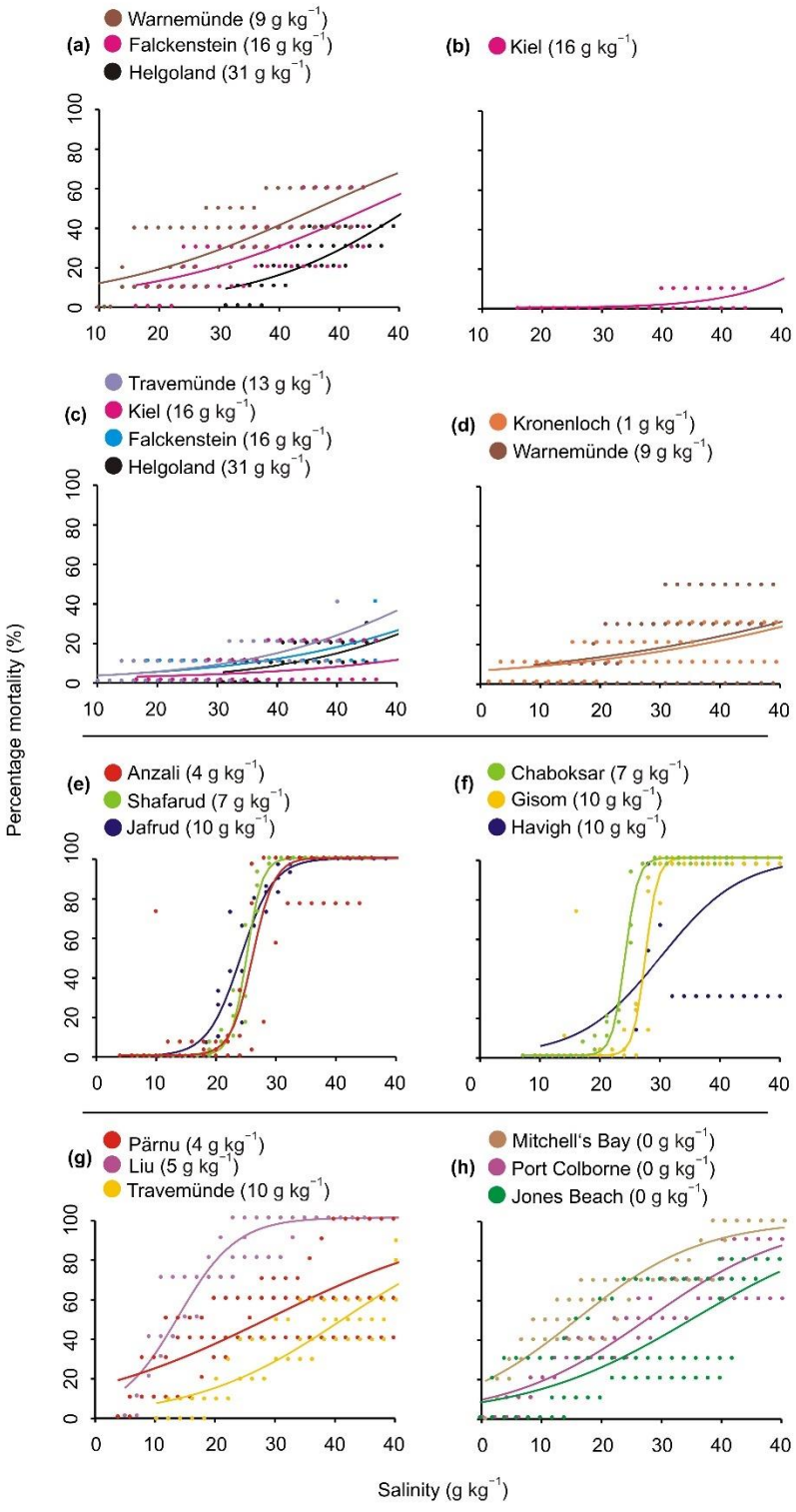

Fig 1. Mortality rates in the increased salinity treatment for Northern European species: (a) *G. locusta*, (b) *G. oceanicus*, (c) *G. salinus* and (d) *G. zaddachi*; Ponto-Caspian species: (e) *P. maeoticus* and (f) *O. crassus*; and Great Lakes–St. Lawrence River species: (g) *G. fasciatus* and (h) *G. tigrinus*. The curves were constructed using pooled data from all three replicates.

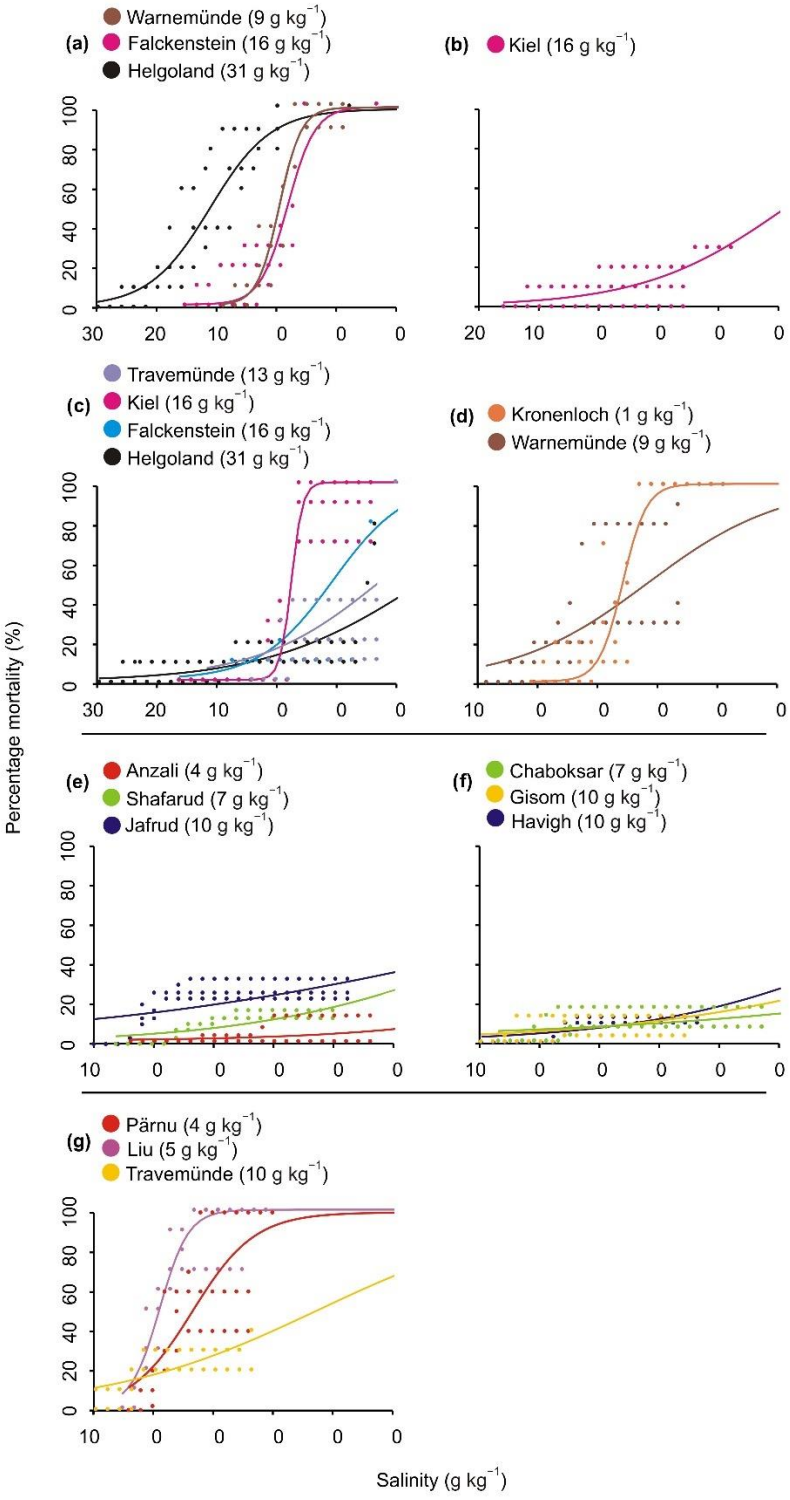

Fig. 2. Mortality rates in the decreased salinity treatment for Northern European species: (a) *G. locusta*, (b) *G. oceanicus*, (c) *G. salinus* and (d) *G. zaddachi*; Ponto-Caspian species: (e) *P. maeoticus* and (f) *O. crassus*; and Great Lakes–St. Lawrence River species: (g) *G. tigrinus*. The curves were constructed using pooled data from all three replicates.

Table 2 Statistical comparisons of parameters between pairs of fitted curves for the populations, which showed significant difference in the onset, rate of mortality, or both.

Species	Population compared	Experimental treatment	The onset of mortality (*P*-value)	The rate of mortality (*P*-value)
Gammarus	Falckenstein – Helgoland	Increase	**0.0099**	0.1220
locusta	Falckenstein – Warnemünde	Increase	0.2415	0.8315
	Helgoland – Warnemünde	Increase	0.0690	0.1349
	Falckenstein – Helgoland	Decrease	**0.0300**	**0.0041**
	Falckenstein – Warnemünde	Decrease	**< 0.0001**	0.3267
	Helgoland – Warnemünde	Decrease	**< 0.0001**	**< 0.0001**
Gammarus	Falckenstein – Helgoland	Increase	0.0772	0.5347
salinus	Helgoland – Kiel	Increase	0.1368	0.5554
	Helgoland – Travemünde	Increase	0.1710	0.9661
	Falckenstein – Kiel	Increase	0.3682	0.8287
	Falckenstein – Travemünde	Increase	0.4914	0.4290
	Kiel – Travemünde	Increase	0.2689	0.5177
	Falckenstein – Helgoland	Decrease	**< 0.0001**	**0.0019**
	Helgoland – Kiel	Decrease	**< 0.0001**	**< 0.0001**
	Helgoland – Travemünde	Decrease	**< 0.0001**	0.3401
	Falckenstein – Kiel	Decrease	**< 0.0001**	**0.001**
	Falckenstein – Travemünde	Decrease	0.0908	0.4914
	Kiel – Travemünde	Decrease	**< 0.0001**	**0.0134**
Gammarus	Warnemünde – Kronenloch	Increase	**0.0002**	0.1774
zaddachi	Warnemünde – Kronenloch	Decrease	**<0.0001**	**0.0003**
Pontogammarus	Jafrud – Shafarud	Increase	**<0.0001**	**<0.0001**
maeoticus	Jafrud – Bandar-e Anzali	Increase	**<0.0001**	0.0960
	Shafarud – Bandar-e Anzali	Increase	**<0.0001**	**0.0102**
	Jafrud – Shafarud	Decrease	0.7971	**0.0333**
	Jafrud – Bandar-e Anzali	Decrease	0.4479	0.5337
	Shafarud – Bandar-e Anzali	Decrease	0.1717	**0.0100**
Obesogammarus	Havigh – Gisom	Increase	**<0.0001**	0.5720
crassus	Havigh – Chaboksar	Increase	**<0.0001**	0.7057
	Gisom – Chaboksar	Increase	0.6910	0.0579

	Havigh – Gisom	Decrease	0.3907	0.4088
	Havigh – Chaboksar	Decrease	**0.0352**	0.0511
	Gisom – Chaboksar	Decrease	0.2362	0.2711
Gammarus tigrinus	Liu – Pärnu	Increase	**<0.0001**	**<0.0001**
	Liu – Travemünde	Increase	**<0.0001**	**<0.0001**
	Pärnu – Travemünde	Increase	**0.0056**	0.0902
	Liu – Pärnu	Decrease	**<0.0001**	**0.0026**
	Liu – Travemünde	Decrease	**<0.0001**	**<0.0001**
	Pärnu – Travemünde	Decrease	**<0.0001**	**0.0006**
Gammarus fasciatus	Mitchell's bay – Port Colborne	Increase	**<0.0001**	**0.0003**
	Mitchell's bay – Jones Beach	Increase	**<0.0001**	0.0573
	Port Colborne – Jones Beach	Increase	**<0.0001**	**0.0138**

The *t*-test incorporated in the Fit Nonlinear Model using Generalized Least Squares was used to test for differences between estimated parameters - rate of mortality (Z_1 and Z_2) and onset of mortality (Q_1 and Q_2). Significant *P*-values are presented in bold. Bonferroni-type protection to guard against inflating the Type I error rate and family-wise error rate of 0.05 were used for pairwise statistical comparisons.

Table 3 Salinity range of the eight tested species based on occurrences in their indigenous and non-indigenous regions.

Species	Salinity Range ppt (indigenous areas)	Salinity Range ppt (non-indigenous areas)
Gammarus locusta	5 to > 30	No invasion range recorded
Gammarus oceanicus	5 to > 30	No invasion range recorded
Gammarus salinus	5 to > 30	No invasion range recorded
Gammarus zaddachi	< 0.5 to > 30	No invasion range recorded
Pontogammarus maeoticus	0.5 to 30	17 - 18
Obesogammarus crassus	12 to 34	< 0.5 to 19
Gammarus tigrinus	< 0.5 to 30	< 0.5 to > 30
Gammarus fasciatus	< 0.5	No invasion range recorded

Evolutionary relationships

A total of 24 sequences (541 base pairs) used for the phylogenetic analyses included 10 species and 24 populations from different salinities. Both methods used to reconstruct phylogeny agreed for the majority of the species-specific clusters (Figure 3). Here, it is important to observe the strong bootstrap support obtained for the nodes of the Neighbor-joining tree that groups G. tigrinus and G. fasciatus (69%), and G. zaddachi and G. salinus (92%). These two clusters are also present in the Bayesian tree and supported by nonoverlapping 95% high probability density intervals in relation to each respective ancestral node.

Discussion

The present study compared the salinity tolerance of eight gammarid species with origin in three different regions—Northern Europe, Ponto-Caspian, and Great Lakes–St. Lawrence River regions—to determine if Ponto-Caspian taxa more readily acclimatize to and colonize diverse salinity habitats than do taxa from other regions. Interestingly, although our study determined that species from all three tested regions tolerate wide ranges of salinity, different patterns arose among species from different regions. Ponto-Caspian species had high survival in fresh water, while Northern European species had high survival in fully marine conditions. Genetic analyses that determined the evolutionary distance among species from different regions, particularly of those from the Ponto-Caspian and the two other regions, aligned with our experimental results. Due to the high tolerance of Ponto-Caspian taxa to freshwater conditions and the evolutionary relationships, in addition to the geological history of the region, we suggest that Ponto-Caspian species are of freshwater origin.

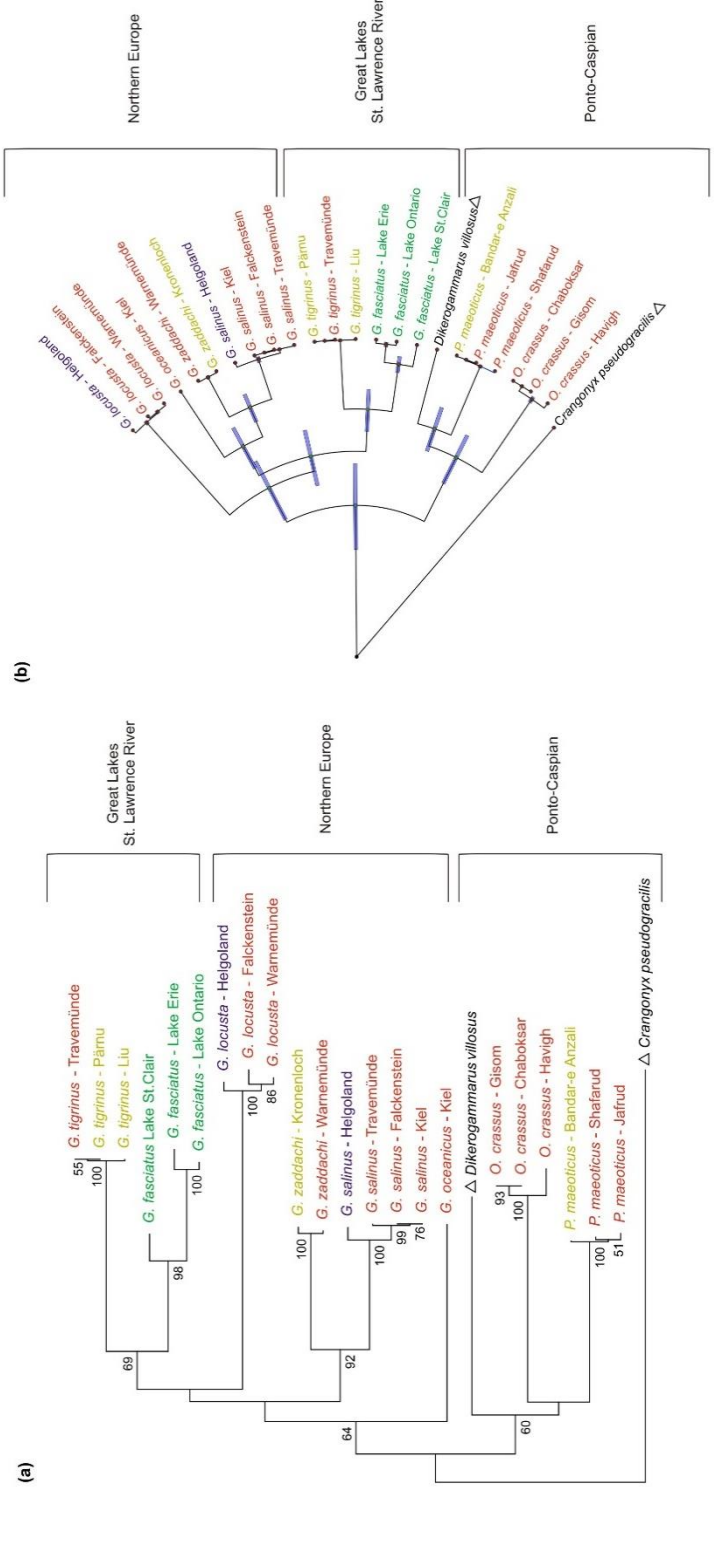

Fig. 3. Evolutionary relationships of taxa: (a) Evolutionary history inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) is shown next to the branches (Felsenstein, 1985). Evolutionary distances were computed using the Tamura 3-parameter method (Tamura, 1992); (b) Evolutionary history inferred through a coalescent-based Bayesian approach. High probability density intervals (HPD) are depicted for each node height. Salinity assignment was based on the Venice System (1958).

Nowadays, Ponto-Caspian species thrive in low salinities of Northern Europe and in the Great Lakes–St. Lawrence River region (Bij de Vaate et al., 2002; Casties et al., 2016; Reid & Orlova, 2002; Ricciardi & MacIsaac, 2000). Geologically, the Ponto-Caspian region has been subject to a number of salinity changes that significantly influenced the biological and physical characteristics of the basins (Zenkevitch, 1963). The species that have persisted and thrived despite these complex changes and successions of low and high salinity levels over millions of years are mostly settled in estuaries, lagoons, and the lower courses of rivers (Mordukhay-Boltovskoy, 1964; Zenkevitch, 1963). From those habitats they might "naturally" migrate northward through rivers/canals to the Baltic Sea, although some of them were intentionally introduced to reservoirs in Russia, Lithuania, and Ukraine (Arbačiauskas, Rakaukas, & Virbickas, 2010; Casties et al., 2016; Jażdżewski, Konopacka, & Grabowski, 2002; Ketelaars, Lambregts-van de Clundert, Carpentier, Wagenvoort, & Hoogenboezem, 1999). Nevertheless, Ponto-Caspian NIS did not spread to more saline habitats of the Black and North Seas, or to the Mediterranean Sea (Paavola, Olenin, & Leppäkoski, 2005; Shiganova, 2010). In contrast, Northern European species tested in our study performed better under marine conditions, confirming their marine origin, which is consistent with the Baltic Sea geological history; the current ecosystem is as recent as the Last Glacial Maximum, that is, <12,000 years ago, and inhabited by Atlantic species (Leppäkoski et al., 2002; Reid & Orlova, 2002). The phylogenetic analyses conducted in our study further supported that evolutionary relationships of these species can be related to their biogeographic regions, as in a fully justified phylogeographic scenario. Fascinatingly, despite the wide salinity tolerance and great number of shipping routes between Northern Europe and other world ports, none of the tested Northern European species have an invasion record. Due to the high tolerance of Ponto-Caspian species to freshwater conditions and Northern European species to high salinity,

in addition to the geological history of the two regions, we hypothesize that these species are of freshwater and marine origins, respectively. Therefore, the establishment of Ponto-Caspian species in the Great Lakes and other freshwater habitats should not be a surprise. However, we advise that investigating the putative molecular basis of adaptation that permits Ponto-Caspian taxa to thrive in fresh water should be done with state-of-the-art techniques, such as high-throughput sequencing, particularly for functional genes responding to salinity conditions.

Species from the same region tested for salinity tolerance demonstrated similar patterns on a large scale, yet they also demonstrated differences among populations on a fine scale. For instance, current literature states that *G. zaddachi* naturally occurs in both freshwater and marine habitats; however, the two populations tested in our study were unable to cope with freshwater conditions even though one of them was collected from salinity of 1 g/kg. Moreover, none of our tested populations of *G. tigrinus*, which has et invaded diverse regions from freshwater to fully marine habitats (Table 3; Kelly al., 2006), perform well either under freshwater or fully marine conditions. This may be the result of different populations of a species adapting to varying local environmental conditions. Several previous studies have indicated that populations may differ significantly in their growth responses to climate change (O'Neill, Hamann, & Wang, 2008), have different tolerance to cold temperatures due to local adaptation (Preisser, Elkinton, & Abell, 2008), and have different upper thermal limits across different latitudes (Kuo & Sanford, 2009). We emphasize that predictions of establishment success, particularly considering future climate change scenarios, might fail as a species is often considered a single homogeneous unit in those predictions instead of diverse locally adapted populations (Crickenberger, Walther, & Moran, 2017; Laurel, Copeman, Spencer, & Iseri, 2017; Parker et al., 2017). While many studies determining invasion success of NIS ask the question: “which traits enable a species to invade a

new habitat?" (Alpert, Bone, & Holzapfel, 2000; Marco, Páez, & Cannas, 2002), we strongly suggest that future studies in both invasion ecology and global change ecology take into consideration spatially varying selection among populations in order to better predict future scenarios.

Both species originating from the Great Lakes–St Lawrence River region did not perform well in our laboratory experiments, often having higher mortality in the controls than in the stressed treatments (Appendix S3). As we regularly observed dark spots on tested animals from these regions, we suspect that the populations were infected with a parasite, most likely oomycetes, also known as water molds. Kestrup, Thomas, van Rensburg, Ricciardi, and Duffy (2011) identified a specific parasitic oomycete as a cause of high mortality of G. fasciatus in its native area of the St. Lawrence River. Interestingly, as the salinity of our experiments was increased, the stressed animals showed lower mortality than those in the control, indicating that the parasite may not have been able to cope with salinity changes. Consequently, if parasites or other disease agents are not able to cope with environmental conditions in a new habitat while the host species is, the effects of the parasite or disease would be reduced in a new region, giving the host a fitness advantage compared to the native region. This parasite or disease loss has been described as the enemy release hypothesis in invasion ecology (Keane & Crawley, 2002). As *G. tigrinus* successfully invaded numerous habitats having a range of salinity and often freshwater areas, one possible explanation for its success may be release from parasites when invading habitats with a different salinity than that of its indigenous area. This enemy release might be short-lived as parasites also might adapt and evolve with time, however, short-term release during the early establishment phase might give enough advantage to facilitate the establishment of the species in a new habitat.

Numerous regions which are geographically far apart have become more similar biologically over recent centuries due to a high exchange of species (Capinha et al., 2015; Hulme, 2009). As Ponto-Caspian species have successfully established in a number of brackish and freshwater habitats in Northern Europe and the Great Lakes–St. Lawrence River region, respectively, but not vice versa, it has been hypothesized that Ponto-Caspian species may have inherent advantages over other species in colonizing new habitats (Casties et al., 2016; Leppäkoski et al., 2002; Ricciardi & MacIsaac, 2000). A major vector for introduction of NIS in aquatic habitats is commercial shipping (Molnar et al., 2008; Ricciardi, 2006), with many shipping ports being located in river mouths and estuaries characterized by broad temporal salinity changes that frequently include freshwater conditions (Keller, Drake, Drew, & Lodge, 2011). As a result, euryhaline taxa might have a great advantage when introduced to those fluctuating environments. Our study suggests that although both Ponto-Caspian and Northern European species tested have quite broad salinity tolerance, possible freshwater origin of relict Ponto-Caspian species might increase establishment success of these taxa due to their tolerance to freshwater conditions. Consequently, Ponto-Caspian species might not be inherently better colonizers, rather, their intrinsic advantage may simply correlate with the fact that areas with greatest introduction frequency of NIS (i.e., shipping ports; Seebens, Gastner, & Blasius, 2013) are environmentally variable habitats which often include freshwater conditions intolerable to euryhaline marine taxa. Finally, although Ponto-Caspian NIS are currently not established in higher salinity areas of the North and Baltic Seas (Casties et al., 2016; Paavola et al., 2005), a future scenario modeled by Meier et al. (2012) predicts a decline in salinity across the Baltic Sea by the end of the twenty-first century, therefore, it may be expected that Ponto-Caspian species will spread further in the system, as well as in other areas with future salinity declines.

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Appendix

APPENDIX S1 List of species, sampling location, salinity of sampling locations, number of sequences, and GenBank accession numbers for specimens collected in this study.

Species	Our samples or downloaded from GenBank	Place of collection (and coordinates if possible)	Salinity of area of collection g kg^{-1}	GenBank accession numbers	References	Sequences used for the trees
Gammarus locusta	Our study	Falckenstein, Germany, 54°39' N 10°19' E	16	KU844859 - KU844872, KU844893 - KU844908, KU844898 - KU844908		KU844872
	Our study	Helgoland, Germany, 54°10' N 07°53' E	31	KU844874 - KU844883, KU844909 - KU844922		KU844869
	Our study	Warnemünde, Germany, 54°13' N 12°09' E	9	KU844884 - KU844892, MF187967 - MF187975	Baltazar-Soares et al. (2017)	MF187967
Gammarus oceanicus	Our study	Kiel, Germany, 54°32' N 10°14' E	16	KU844923 - KU844949		KU844947
Gammarus salinus	Our study	Kiel, Germany, 54°33' N 10°15' E	16	KU844959 - KU844967 KU844979 - KU844983		KU844968
	Our study	Falckenstein, Germany, 54°40' N 10°20' E	16	KU844968 - KU844978		KU844971
	Our study	North Beach, Helgoland,Germany, 54°11' N 07°52' E	31	MF187976 - MF187985 KU844979 - KU844981; KU844984	Baltazar-Soares et al. (2017)	MF187981

	Our study	Travemünde, Germany, 53°83' N 10°64' E	13	KU844979 - KU844981 KU844984, MF187986 - MF187999	Baltazar-Soares et al. (2017)	MF187986
Gammarus zaddachi	Our study	Warnemünde, Germany, 54°13' N 12°10' E	9	KU845051 - KU845069, MF188009 - MF188014	Baltazar-Soares et al. (2017)	MF188009
	Our study	Kronenloch, Germany, 54°09' N 8°96' E	1	KU845070 - KU845095		KU845072
Gammarus tigrinus	Our study	Travemünde, Germany, 53°83' N 10°64' E	10	KU844997 - KU845006		KU845006
	Our study	Liu, Estonia, 58°22' N 24°28' E	4.7	KU845007 - KU845027, MF188005 - MF188008	Baltazar-Soares et al. (2017)	KU845015
	Our study	Pärnu, Estonia, 58°36' N 24°46' E	4.28	KU845028 - KU845050, MF188000 - MF188004	Baltazar-Soares et al. (2017)	MF188000
Gammarus fasciatus	Our study	Jones Beach, Port Weller, Lake Ontario, Canada, 43°13' N 79°12' W	< 0.5	MG734961, MG754480 - MG754484, MG754497 - MG754501		MG734961
	Our study	Port Colborne, Lake Erie, Canada, 42°52' N 79°12' W	< 0.5	MG734961 - MG734965, MG754485 - MG754490		MG734965
	Our study	Mitchell's Bay, Lake St. Clair, Canada, 42°28' N 82°26' W	< 0.5	MG734966 - MG734968, MG754491 - MG754496		MG734966
Obesogammarus crassus	Our study	Gisom, Iran, 37°51' N 48°58' E	10.5	KU845096 - KU845099, KU845108- KU845110, MF188015 - MF188021	Baltazar-Soares et al. (2017)	MF188015

	Our study	Havigh, Iran, 38°09' N 48°55' E	10.5	KU845100 - KU845107, MF188022 - MF188030	Baltazar-Soares et al. (2017)	MF188022
	Our study	Chaboksar, Iran, 36°60' N 50°34' E	9	MF188031 - MF188038	Baltazar-Soares et al. (2017)	MF188031
Pontogammarus maeoticus	Our study	Shafarud, Iran, 38°21' N 48°52' E	7.2	KU845119 - KU845122, KU845127, KU845128, KU845156 - KU845160, MF188060 - MF188071	Baltazar-Soares et al. (2017)	MF188060
	Our study	Jafrud, Iran, 37°37' N 49°07' E	4.3	KU845115 - KU845118, KU845125, KU845126, KU845129 - KU845142, MF188052 - MF188059	Baltazar-Soares et al. (2017)	KU845124
	Our study	Bandare Anzali, Iran, 37°29' N 49°30' E	10.6	KU845111- KU845114, KU845123, KU845124, KU845143 - KU845155, MF188039 - MF188051	Baltazar-Soares et al. (2017)	MF188064
Crangonyx pseudogracilis	GenBank			EF570296	Hou and Sket (2016)	EF570296
Dikerogammarus villosus	GenBank			EF570297	Hou and Sket (2016)	EF570297

APPENDIX S2 References used to compile the range of salinity for indigenous and nonindigenous occurrences of the eight species tested (i.e., Table 3).

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APPENDIX S3 Statistical comparisons of parameters between pairs of fitted curves of the increase/decrease and control treatments within populations, which showed significant difference in the onset, rate of mortality, or both.

Species	**Population compared**	**Experimental treatment**	**The onset of mortality (*P*-value)**	**The rate of mortality (*P*-value)**
Gammarus locusta	Falckenstein – Falckenstein	Increase – Control	**0.0290**	0.6405
	Helgoland – Helgoland	Increase – Control	**0.0002**	0.5167
	Warnemünde – Warnemünde	Increase – Control	0.1563	0.5918
	Falckenstein – Falckenstein	Decrease – Control	**< 0.0001**	**0.0001**
	Helgoland – Helgoland	Decrease – Control	**< 0.0001**	**< 0.0001**
	Warnemünde – Warnemünde	Decrease – Control	**< 0.0001**	**< 0.0001**
Gammarus oceanicus	Kiel – Kiel	Increase – Control	0.2351	**0.0921**
	Kiel – Kiel	Decrease – Control	0.3474	0.2280
Gammarus salinus	Falckenstein – Falckenstein	Increase - Control	0.5424	0.2022
	Helgoland – Helgoland	Increase - Control	**0.0230**	0.1773
	Kiel – Kiel	Increase - Control	0.5331	0.3957
	Travemünde – Travemünde	Increase - Control	0.7804	0.4484
	Falckenstein – Falckenstein	Decrease - Control	**0.0013**	**<0.0001**
	Helgoland – Helgoland	Decrease - Control	**0.0251**	**0.0023**
	Kiel – Kiel	Decrease - Control	**<0.0001**	**<0.0001**

	Travemünde – Travemünde	Decrease - Control	**<0.0001**	**<0.0001**
Gammarus zaddachi	Warnemünde – Warnemünde	Increase - Control	0.0670	**0.0447**
	Kronenloch – Kronenloch	Increase - Control	0.0742	0.7944
	Warnemünde – Warnemünde	Decrease - Control	**<0.0001**	0.2768
	Kronenloch – Kronenloch	Decrease - Control	**<0.0001**	**<0.0001**
Gammarus tigrinus	Liu – Liu	Increase - Control	0.1161	0.3382
	Pärnu – Pärnu	Increase - Control	**<0.0001**	**0.0004**
	Travemünde – Travemünde	Decrease - Control	**<0.0001**	**0.0300**
	Liu – Liu	Decrease - Control	**<0.0001**	**0.0006**
	Pärnu – Pärnu	Decrease - Control	**<0.0001**	**0.0001**
	Travemünde – Travemünde	Decrease - Control	**0.0012**	0.7968
Gammarus fasciatus	Mitchell's bay – Mitchell's bay	Increase - Control	**<0.0001**	**<0.0001**
	Port Colborne – Port Colborne	Increase - Control	**<0.0001**	**0.0040**
	Jones Beach – Jones Beach	Increase - Control	**<0.0001**	**0.0001**

Chapter 3

Are juveniles as tolerant to salinity stress as adults? A case study of Northern European, Ponto-Caspian and North American species

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Filipa Paiva [1,2], Nora-Charlotte Pauli[3] and Elizabeta Briski[1]

[1]GEOMAR, Helmholtz-Zentrum für Ozeanforschung Kiel, Düsternbrooker Weg 20, 24105 Kiel, Germany

[2]MARE – Marine and Environmental Sciences Centre, Quinta do Lorde Marina, Sítio da Piedade, 9200-044, Caniçal, Madeira Island, Portugal

[3]Carl-von-Ossietzky Universität Oldenburg, Institute for Chemistry and Biology of the Marine Environment, Carl-von-Ossietzky-Str. 9-11, 26129 Oldenburg, Germany

Abstract

Aim: Global biodiversity and ecosystems are highly impacted by anthropogenic activities, such as climate change and introduction of non-indigenous species. As numerous species from the Ponto-Caspian region have established in the North and Baltic Seas, as well as in the Laurentian Great Lakes, there have been large number of studies examining environmental tolerance of these species to determine their future potential to spread. However, many of those studies were conducted only on adult stages, while neglecting the possibility that early life history stages might not be equally resilient.

Location: Northern European, Ponto-Caspian and North American regions

Methods: To determine if juveniles would demonstrate the same environmental tolerance as their parents, we examined the salinity tolerance of adults and juveniles of one Northern European (*Gammarus salinus*), one Ponto-Caspian (*Pontogammarus maeoticus*) and one North American species (*Gammarus tigrinus*). Additionally, we compared our study to that of Paiva et al. (2018), who tested the salinity tolerance of the same species using only adults.

Results: Our study determined that both adults and juveniles of all three species tolerated wide ranges of salinity, with juveniles of *G. salinus* tolerating only slightly narrower salinity range than their parents, while those of *P. maeoticus* and *G. tigrinus* much narrower range. Additionally, we determined better survival and higher growth rates of juveniles of *G. salinus* in higher salinities, and better survival of *P. maeoticus* in lower salinities.

Main conclusions: Based on juvenile salinity tolerance, our study further supported findings of Paiva et al. (2018), where Northern European species may be adapted to marine, while Ponto-Caspian to lower saline and freshwater environments. The North American species is probably adapted to intermediate salinities. As juveniles do not tolerate the same salinity stress as adults,

we emphasize the importance of testing all life history stages when predicting species resilience to environmental stressors.

Keywords: early life-history stages, *Gammarus salinus, Gammarus tigrinus,* growth rate, hatching success, juveniles, non-indigenous species, *Pontogammarus maeoticus*, salinity tolerance

Introduction

Marine and freshwater ecosystems are largely affected by anthropogenic stressors like eutrophication, pollution, habitat loss, climate change, and biological invasions (Capinha, Essl, Seebens, Moser, & Pereira, 2015; Chapman, 2017; Lockwood, Hoopes, & Marchetti, 2013; Solan & Whiteley, 2016), all of which are a threat to global biodiversity. The introduction and establishment of non-indigenous species (NIS) via human-mediated transport can have strong impacts on marine biodiversity and ecosystem structure, altering communities worldwide (Simberloff, 2011; Strayer, Eviner, Jeschke, & Pace, 2006). Successful establishment of a NIS requires a sufficient number of viable and reproductively capable individuals (i.e., propagule pressure), certain species characteristics (e.g., phenotypic plasticity), and it also depends on the conditions of the recipient habitat (i.e., environmental conditions and interaction of NIS with native species; Lockwood et al., 2013; Ruiz, Carlton, Grosholz, & Hines, 1997; Simberloff, 2009). Recently, Briski et al. (2018) suggested that selection during the transport stage of the invasion process can facilitate local adaptation (e.g., survival of only pre-adapted individuals for particular environmental conditions), which may result in greater likelihood of invasion success. Likewise, several studies have suggested that certain geographical regions are major donors of NIS, in particular those with disturbed geological history and environmental fluctuations that have led to selection for flexible life history traits, phenotypic plasticity and consequently more robust species

(Bij de Vaate, Jażdżewski, Ketelaars, Gollasch, & Van der Velde, 2002; Casties, Seebens, & Briski, 2016; Reid & Orlova, 2002; Ricciardi & MacIsaac, 2000).

The Ponto-Caspian region (i.e., Black, Azov and Caspian Seas) has been determined as one of the major sources of NIS to different types of water bodies, including brackish and freshwater habitats of Northern Europe and the Laurentian Great Lakes (Bij de Vaate et al., 2002; Casties et al., 2016; Mordukhay-Boltovskoy, 1964; Reid & Orlova, 2002; Ricciardi & MacIsaac, 2000). As only a small number of species from the Great Lakes invaded Northern European waters and vice versa, several studies suggested that Ponto-Caspian taxa more readily colonize habitats of diverse salinities than taxa from other regions (Leppäkoski et al., 2002; Reid & Orlova, 2002; Ricciardi & MacIsaac, 2000). The Ponto-Caspian basin is geologically old and was continuously affected by large-scale environmental fluctuations from fully marine environments, as a part of the Tethys Sea, to almost pure freshwater ecosystems as Sarmatian Sea (Reid & Orlova, 2002; Zenkevitch, 1963). Considering these hydrological changes, many Ponto-Caspian species have been selected for euryhalinity (Reid & Orlova, 2002). In addition, some studies suggested that Ponto-Caspian NIS, established in freshwater habitats, might not be of marine, but of freshwater origin due to the geological history of their native region (Casties et al., 2016; Paiva et al., 2018; Reid & Orlova, 2002). To support this hypothesis, there has been an increasing number of studies investigating the salinity tolerance of Ponto-Caspian species distributed in brackish and freshwater habitats (e.g., Dobrzycka-Krahel & Graca, 2018; Kobak et al., 2017; Paiva et al., 2018). Recently, Pauli and Briski (2018) conducted an extensive literature search on the salinity range of Ponto-Caspian NIS in their native and non-native habitats and determined that though Ponto-Caspian species occupy wide ranges of salinity, more than 67% of the species were recorded in freshwater habitats in their native region, with a tendency of a decreasing number of species as salinity

increased. The similar evidence was provided by Pauli, Paiva, and Briski (2018) demonstrating that artificial selection of one Ponto-Caspian gammarid, originating from a salinity of 10 g/kg, is possible to lower salinities and freshwater conditions, but not to higher salinities. Finally, a comparative salinity assessment, using adults of 22 populations of eight gammarid species originating from the Ponto-Caspian, Northern European and Great Lakes–St. Lawrence River regions, revealed that Ponto-Caspian taxa performed better in freshwater, while Northern European taxa performed better in fully marine conditions (Paiva et al., 2018).

In terms of geographical range expansions and biological invasions, it was assumed that salinity would limit species dispersal from marine to brackish and freshwater habitats, and vice versa (Dahl, 1956). However, numerous studies have reported the establishment of marine and brackish species in freshwater habitats, with many of those species originating from the Ponto-Caspian basin (Casties et al., 2016; Lee & Bell, 1999; Pauli & Briski, 2018; Ricciardi & MacIsaac, 2000; Ruiz et al., 1997). As with most environmental stressors, salinity stress often more severely affects early life history stages, such as embryos and larvae, than adults (e.g., Anger, 2003; Kinne, 1964). The osmotic stress encountered when salinity limits are exceeded requires energetic costs that may not only compromise major physiological needs, but also have negative consequences on reproduction, development, growth and survival of stressed individuals (Anger, 2003; Neuparth, Costa, & Costa, 2002; Normant & Lamprecht, 2006). Even though adult organisms can tolerate a wide range of salinities, they may not be able to reproduce (Steele & Steele, 1991 and references therein), or when they do, the stress may have severe consequences for their offspring, such as reduced viability of embryos, decreased number of broods and reduced number of emergent juveniles (e.g., Mills & Fish, 1980; Steele & Steele, 1991; Vlasblom & Bolier, 1971). Finally, even when individuals of an introduced population are able to survive and reproduce, they can still fail

to establish in a new habitat if the population growth rate is negative, which particularly may be the case when small populations are introduced (Blackburn et al., 2011).

Although salinity tolerance has been studied for different species globally (e.g., Dobrzycka-Krahel & Graca, 2018; Ellis & MacIsaac, 2009; Kobak et al., 2017; McFarland, Baker, Baker, Rybovich, & Volety, 2015; Ovčarenko, Audzijonyte, & Gasinjnaite, 2006; Paiva et al., 2018), it remains unclear how offspring would respond to those salinities. In this study, we extend the comparative salinity assessment of Paiva et al. (2018) by evaluating not only adults, but also juveniles of one Northern European (*Gammarus salinus*), one Ponto-Caspian (*Pontogammarus maeoticus*) and one North American species (*Gammarus tigrinus*) to determine whether adults and juveniles would reveal the same salinity pattern (Figure 1). The Northern European and Ponto-Caspian species were collected in their native range, while the North American species was collected in its invaded range, due to practicality and laboratory proximity. However, we emphasize that the aim of this study was not to compare populations from native and introduced locations of the same species, but to compare performance between adults and juveniles of species originating from different regions. To evaluate fitness of the tested species, we exposed pairs in precopula to different salinities and followed mortality of adults, and hatching success, growth rate and mortality of juveniles. We tested the hypotheses that there is no difference in (a) mortality of adults among different treatments and species; (b) mortality of juveniles among different hatching salinities and species; and (c) growth rate of juveniles among different hatching salinities and species. Additionally, we compared our results to those in Paiva et al. (2018) and tested the hypotheses that there is no difference in mortalities: (d) of adults between the two studies; and (e) of juveniles in this study and adults in Paiva et al. (2018).

Materials and methods

Specimen collection

Specimens of *P. maeoticus* were collected in October 2014 in Jafrud, Iran (37°37' N 49°07' E), of *G. tigrinus* in May 2016 in Travemünde, Germany (53°83' N 10°64' E), and of *G. salinus* in May 2017 in Falckenstein, Germany (54°40' N 10°20' E). Two species were collected in their native range (i.e., *G. salinus* and *P. maeoticus*), and one in its non-native region (i.e., *G. tigrinus*; Fig. 1). While the perfect scenario would be to have all three species collected in its native range, *G. tigrinus* was collected in its invaded location due to practicality and laboratory proximity. After collection, individuals were transported in ambient water to the laboratories at GEOMAR in Kiel, Germany, where each individual was morphologically identified according to Köhn and Gosselck (1989) for *G. salinus*; Sars (1896), Birstein and Romanova (1968), Moiceiev and Filatova (1985), Stock (1974) and Stock, Mirzajani, Vonk, Naderi, and Kiabi (1998) for *P. maeoticus*; and Lincoln (1979) for *G. tigrinus*. Before experiments started, animals were kept at their ambient salinity for at least two weeks to acclimatize to laboratory conditions; we emphasize that in the case of *P. maeoticus* the tested population was kept in the laboratory for 1.5 years before the experiments started.

Laboratory experiments

To evaluate fitness of each species, we exposed adult individuals to different salinities, and followed their mortality, as well as hatching success, growth rate and mortality of juveniles. The experiments were conducted from April 2016 to June 2017. The experimental design for adults consisted of three treatments: a) control; b) low salinity; and c) high salinity. Each treatment consisted of five replicates (i.e., five 2 L tanks). Five pairs of mean-size individuals in precopula (i.e., male holding on to and carrying female) were placed in each 2 L tank. In the case of *P.*

maeoticus there were not enough couples available; therefore, in each tank three couples and four randomly chosen single adult individuals were used. Seawater filtered through a 20 µm mesh from Kiel Fjord (fluctuating from 10 g/Kg to 16 g/Kg) was used for the experiments, which salinity was then increased and decreased using artificial seawater (Instant Ocean®) and potable tap water, respectively to reach the desired salinity for each treatment. The salinity of the control treatments

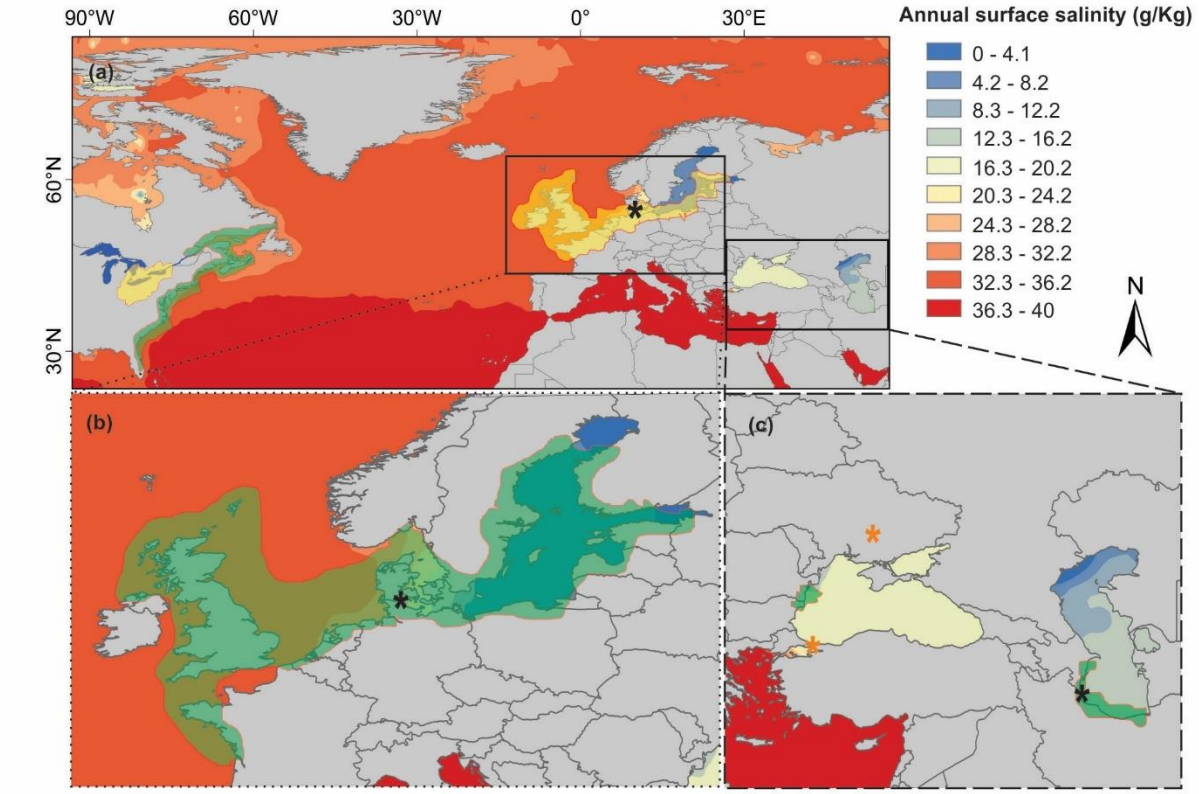

Fig. 1. Geographic range and sampling locations of G. tigrinus (a), G. salinus (b) and P. maeoticus (c). Native and invaded ranges of G. tigrinus are shown in green and yellow, respectively; native range of G. salinus is shown in green; and native and invaded ranges of P. maeoticus are shown in green and by the yellow circle, respectively. Black asterisks denote sampling locations in our study.

was identical to ambient water of the collection site for each species: for *G. salinus* 16 g/Kg, for *P. maeoticus* 10 g/Kg, and for *G. tigrinus* 10 g/Kg. The high and low salinity treatments began at the ambient salinity of the species collection site, which was then increased/decreased by 2 g/Kg

every two days, until reaching 40 g/Kg and 0 g/Kg, respectively (Delgado et al., 2011; Paiva et al., 2018; Pauli et al., 2018). Therefore, we emphasize here that due to the different ambient salinities of the three species, the high and low treatments did not start from the same salinities for all three species. Salinity was increased/decreased by removing half of the water in the tanks and replacing it with in advance prepared water of the required salinity; water of the required salinities was prepared seven days in advance to allow for proper dissolving of artificial salt (Instant Ocean®). Salinity was measured using a WTW Cond 3110 salinometer and a Tetracon 325 probe (Xylem Analytics Germany Sales GmbH & Co. KG, WTW, Germany). The accuracy of the desired salinities in the experiments was ± 0.2 g/Kg. As 1 L of water was immediately poured into the tanks, to apply the same disturbance/stress to the control treatment, water was also exchanged every two days. We emphasize that this way of water change may be an additional cause of mortality. Mortality of adults was checked daily throughout the experiments. When 40 g/Kg and 0 g/Kg were reached, mortality of adults was followed for two more weeks before the experiment was terminated.

Before each water exchange (i.e., every second day), tanks were examined thoroughly for the presence of a new cohort of juveniles. If found, the juveniles were removed using a pipette, and placed in a new 2 L tank where they were kept for six weeks to allow us to follow their growth rates. The juveniles of different age cohorts were placed in separate tanks. Juveniles from two cohorts, corresponding to two salinity steps, were reared together at the intermediate salinity of two salinity steps. For example, juveniles hatched at 8 g/Kg and 6 g/kg were reared together at 7 g/Kg. Once juveniles were placed in the rearing tanks of a salinity that was 1 g/Kg higher or lower than their hatching salinity, salinity of the tanks was not changed anymore during the six-week experiment. Following the experimental design of adults, juveniles were also reared in five

replicates corresponding to the tank numbers of adults. The water in the rearing tanks was exchanged weekly, and the juveniles were fed *ad libitum* with fish food flakes. Hatching success was determined as the total number of juveniles per cohort, including dead individuals. Juvenile mortality and growth rate were checked every two weeks. Growth rate was determined by measuring the cephalon length using a stereomicroscope (Stemi 508, ZEISS) and the ZEN software (vs. 2.3, ZEISS), where a juvenile was placed in a droplet of water on a microscope slide and gently covered by a cover slip to restrain movements of the animal. The cephalon length was used as a proxy for total length to minimize handling and stressing the animals (Delgado et al., 2011; Lancellotti and Trucco, 1993). Dead animals were not measured.

Statistical Analysis

To determine the effect of salinity on mortality of adults, we tested for differences in the onset and rate of mortality between treatments within species, between species, and between our study and Paiva et al. (2018). A mortality curve for each treatment for each species was created using all replicates, described by the equation (Briski, Ghabooli, Bailey, & MacIsaac, 2011; Briski, VanStappen, Bossier, & Sorgeloos, 2008):

$$y=100/[1+e\text{-}Z(s\text{-}Q)] \quad (1)$$

where s is salinity change, Z is the rate of mortality and Q is the onset of mortality. The model was then expanded to compare the rate and the onset of mortality between two curves using the equation:

$$y=100/[1+e\text{-}(Z_1+Z_2)(s\text{-}Q_1\text{-}Q_2)] \quad (2)$$

where Z_1 and Z_2 were the rates of mortality, and Q_1 and Q_2 were the points of onset of mortality, for the first and second curve, respectively. All possible combinations of curve pairs were compared statistically by the Fit Nonlinear Model using Generalized Least Squares. Significant levels for statistical comparisons of estimated parameters Z_1 and Z_2, and Q_1 and Q_2, were adjusted for multiple pairwise comparisons by Bonferroni-type correction to guard against inflating the Type I error rate and the family-wise error rate of 0.05 was used. The analyses were performed using S-Plus 6.1 (S-Plus® 6.1, 2002, Insightful Corp., Seattle, Washington, USA). Additionally, mortalities among three species at the end of the experiment were compared using three one-way ANOVAs, each for one treatment (i.e., control, low and high salinity treatment). Post-hoc pairwise comparisons using Tukey HSD test were also performed. The assumptions of parametric tests were fulfilled.

In the case of juveniles, again for all comparisons, the assumptions of normality and homogeneity of variances were checked, and based on the obtained results the decision on the type of test – parametric or nonparametric – was made. The effect of salinity on mortality of juveniles was tested using Kruskal-Wallis H-test. Three separate Kruskal-Wallis H-tests were conducted, each for one species. Additional post-hoc pairwise comparisons using Wilcoxon rank sum test with Bonferroni adjustment were also performed. The effect of salinity on the cephalon length of juveniles of *G. salinus* was also conducted using Kruskal-Wallis H-test, with an additional post-hoc pairwise comparison using a Wilcoxon rank sum test with Bonferroni adjustment. To test for the effect of salinity on the cephalon length of *P. maeoticus* and *G. tigrinus*, two separate one-way ANOVAs were done, each for one species. Additional post-hoc pairwise comparisons using Tukey HSD test were performed. The salinities at which no juveniles survived until the end of the

experiment were excluded from the cephalon length analyses (i.e., 2 out of 11 salinities for *G. salinus*, 4 out of 9 salinities for *P. maeoticus*, and 3 out of 9 salinities for *G. tigrinus*). The tanks were used as replicates in all statistical comparisons. All statistical comparisons used data from the end of the experiments. Statistical analyses were performed using R software, version 3.5.2 (R Core Team, 2018). Data visualization was conducted by "ggpubr" and "ggplot2" packages in R (Kassambara, 2018; Wickham, 2016).

Results

Mortality of adults

In general, all three species demonstrated a wide range of salinity tolerance. Interestingly, the highest differences were observed in the control treatment where adults of *G. tigrinus* started to die significantly earlier and with a significantly faster mortality rate than those of *G. salinus* and *P. maeoticus* (Table 1; Fig 2). Though the onset of mortality of *G. salinus* was earlier than that of *P. maeoticus*, there was no difference in the mortality rate between the two species (Table 1; Fig 2). Consequently, at the end of the experiments, the mortality of *G. tigrinus* was the highest (94%), followed by that of *G salinus* (58%), and then by that of *P. maeoticus* (24%; ANOVA, $F(2, 12) = 55.7$, $p < 0.0001$; TUKEY HSD: *G. salinus* – *P. maeoticus* $p = 0.0006$, *G. tigrinus* – *P. maeoticus* $p < 0.0001$, *G. tigrinus* – *G. salinus* $p = 0.0004$; Fig. 2). In the low salinity treatment, adults of *G. salinus* started to die significantly later than those of *P. maeoticus* and *G. tigrinus*, though at similar salinities (Table 1; Fig. 2). The mortality rate was similar among the three species (Table 1; Fig. 2). At the end of the experiments, there was no significant difference in the mortalities among the species (70%, 54% and 62% for *G. salinus*, *P. maeoticus* and *G. tigrinus*, respectively; ANOVA, $F(2, 12) = 1.28$, $p = 0.313$; Fig. 2). Finally, in the high salinity treatment, there were significant differences in the onset and rate of mortality among all three species (Table 1; Fig. 2). At the end

of the experiments, there was a difference in the mortalities between *G. salinus* and *P. maeoticus*, but not between *G. salinus* and *G. tigrinus*, nor between *P. maeoticus* and *G. tigrinus* (84%, 100% and 96% for *G. salinus*, *P. maeoticus* and and *G. tigrinus*, respectively; ANOVA, $F(2, 12) = 1.28$, $p = 0.313$; Fig. 2). Finally, in the high salinity treatment, there were significant differences in the onset and rate of mortality among all three species (Table 1; Fig. 2). At the end of the experiments, there was a difference in the mortalities between *G. salinus* and *P. maeoticus*, but not between *G. salinus* and *G. tigrinus*, nor between *P. maeoticus* and *G. tigrinus* (84%, 100% and 96% for *G. salinus*, *P. maeoticus* and *G. tigrinus*, respectively; ANOVA, $F(2, 12) = 6.5$, $p = 0.0122$; TUKEY HSD: *G. salinus* – *P. maeoticus* $p = 0.0120$, *G. tigrinus* – *P. maeoticus* $p = 0.6708$, *G. tigrinus* – *G. salinus* $p = 0.0565$; Table 1; Fig. 2). *Gammarus tigrinus* started to die significantly faster than *G. salinus*, while *P. maeoticus* started significantly later than the other two species (Table 1; Fig. 2). However, the mortality rate of *P. maeoticus* was the fastest, followed by that of *G. salinus* and then by that of *G. tigrinus* (Table 1; Fig. 2).

When mortalities of adults were compared among treatments, *G. tigrinus* performed the worst in the control treatment, while *P. maeoticus* was the worst in the high salinity treatment (Table 2; Fig. 2). In the case of *G. salinus*, there were no extreme differences among the treatments, though some of them were significant (Table 2; Fig 2). The adults of *G. salinus* started to die significantly earlier in the high salinity treatment, with a faster mortality rate than those in the control and low salinity treatments (Table 2; Fig 2). Though the onset of mortality in the control and low salinity treatments was similar, the mortality rate in the low salinity treatment was faster than that in the control (Table 2; Fig 2). In the case of *P. maeoticus*, the adults first started to die in the low salinity treatment, followed by the high salinity treatment and then by the control, with a faster mortality rate in the high salinity treatment compared to the low; the rate of mortality in

the control treatment was the slowest (Table 2; Fig. 2). Finally, the onset of mortality and mortality rate of *G. tigrinus* were significantly later and slower in the low salinity treatment than those in the control and high salinity treatments (Table 2; Fig 2).

Table 1 Statistical comparisons of parameters between pairs of fitted curves for mortality of adults between species.

Species compared	**Experimental treatment**	**The onset of mortality (*P*-value)**	**The rate of mortality (*P*-value)**
Gammarus salinus – *Pontogammarus maeoticus*	Control	**< 0.0001**	0.0873
	Low	**0.0133**	0.3701
	High	**< 0.0001**	**< 0.0001**
Gammarus salinus – *Gammarus tigrinus*	Control	**< 0.0001**	**< 0.0001**
	Low	**0.0015**	0.4050
	High	**0.0009**	**0.0056**
Pontogammarus maeoticus – *Gammarus tigrinus*	Control	**< 0.0001**	**< 0.0001**
	Low	0.1357	0.4402
	High	**0.0013**	**< 0.0001**

The Fit Nonlinear Model using Generalized Least Squares was used to test for differences between estimated parameters Z_1 and Z_2, and Q_1 and Q_2. Significant *P*-values are presented in bold. Bonferroni-type protection to guard against inflating the Type I error rate and family-wise error rate of 0.05 were used for pairwise statistical comparisons.

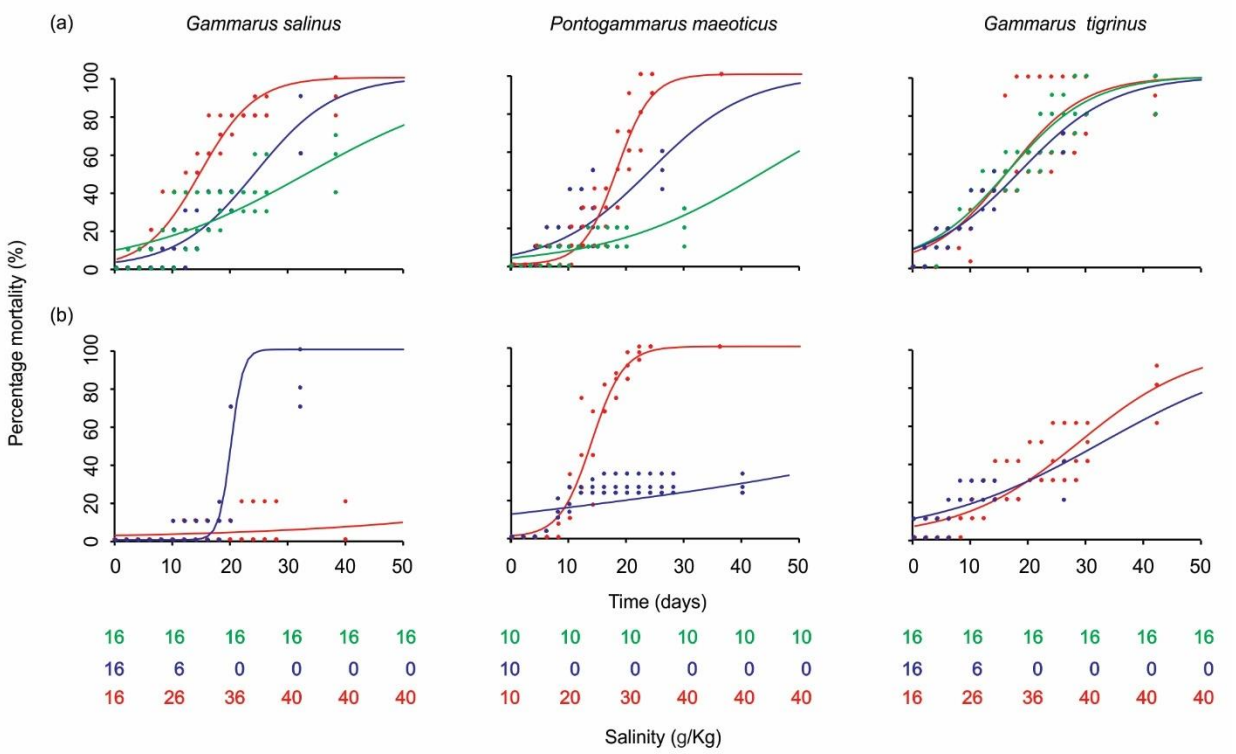

Fig. 2. Mortality rates of adults of *G. salinus*, *P. maeoticus* and *G. tigrinus* in our study (a) and in Paiva et al. (2018) (b). Experimental treatments are depicted in green, blue and red for control, low and high salinity treatment, respectively. The green blue and red numbers symbolize the respective salinity changes over time for different treatments. Note that Paiva et al. (2018) did not report controls. The curves were constructed using pooled data from all replicates.

Table 2 Statistical comparisons of parameters between pairs of fitted curves for mortality of adults between treatments.

Species	Experimental treatment	The onset of mortality (*P*-value)	The rate of mortality (*P*-value)
Gammarus salinus	Control – High	**< 0.0001**	**< 0.0001**
	Control – Low	0.6194	**0.0264**
	High – Low	**< 0.0001**	**0.0011**
Pontogammarus maeoticus	Control – High	**< 0.0001**	**< 0.0001**
	Control – Low	**< 0.0001**	**< 0.0001**
	High – Low	**< 0.0001**	**< 0.0001**
Gammarus tigrinus	Control – High	0.3489	0.4844
	Control – Low	**< 0.0001**	**0.0077**
	High – Low	**0.0009**	**0.0056**

Note: The Fit Nonlinear Model using Generalized Least Squares was used to test for differences between estimated parameters Z_1 and Z_2, and Q_1 and Q_2. Significant *P*-values are presented in bold. Bonferroni-type protection to guard against inflating the Type I error rate and family-wise error rate of 0.05 were used for pairwise statistical comparisons.

Comparison of mortality of adults in our study with that in Paiva et al. (2018)

While Paiva et al. (2018) clearly determined a high tolerance of *G. salinus* and *P. maeoticus* in the high and low salinity treatments, respectively, our study did not confirm the same tolerance (Table 3; Fig. 2). Yet, a low tolerance of these two species was confirmed for the low and high salinity treatments, respectively (Table 3; Fig. 2). In the case of *G. salinus*, adults in the low salinity treatment had a significantly faster mortality rate in our study than in Paiva et al. (2018), but with the same onset of mortality (Table 3; Fig. 2). In the high salinity treatment, adults started to die significantly earlier in our study, with a significantly faster mortality rate (Table 3; Fig. 2). While, the mortality in the low salinity treatment at the end of the experiments was lower in our study than in Paiva et al. (2018) (70% and 87%, respectively), the opposite was observed in the high salinity treatment (84% and 25%, respectively; Table 3; Fig. 2). In contrast to *G. salinus*, there

was no difference between the two studies in the mortality rate of *P. maeoticus* in the high salinity treatment, but there were significant differences, with significantly faster onset and mortality rate in the low salinity treatment in our study compared to Paiva et al. (2018; Table 3; Fig. 2). In our study, the mortality in the low salinity treatment was almost double than that in Paiva et al. (2018) (54% and 29%, respectively; Table 3; Fig. 2). In the case of *G. tigrinus*, our study revealed a significantly earlier onset and faster mortality rate in the high salinity treatment compared to those in Paiva et al. (2018) (Table 3; Fig. 2). The mortalities in our study were also higher in both the low (62% and 53% in our study and Paiva et al. (2018), respectively) and high salinity treatments (96% and 77%, respectively; Table 3; Fig. 2).

Table 3 Statistical comparisons of parameters between pairs of fitted curves for mortality of adults between our study and Paiva et al. (2018).

Species compared	Experimental treatment	The onset of mortality (*P*-value)	The rate of mortality (*P*-value)
Gammarus salinus	Low	0.0551	< **0.0001**
	High	< **0.0001**	< **0.0001**
Pontogammarus maeoticus	Low	< **0.0001**	< **0.0001**
	High	< **0.0001**	0.6845
Gammarus tigrinus	Low	0.1357	0.4402
	High	< **0.0001**	**0.0004**

Note: The control treatments were not compared as Paiva et al. (2018) did not report controls. The Fit Nonlinear Model using Generalized Least Squares was used to test for differences between estimated parameters Z_1 and Z_2, and Q_1 and Q_2. Significant *P*-values are presented in bold. Bonferroni-type protection to guard against inflating the Type I error rate and family-wise error rate of 0.05 were used for pairwise statistical comparisons.

Hatching success and mortality of juveniles

In general, all three species were able to reproduce across different salinities, but experienced mortality of juveniles throughout the six-week experimental period (Fig. 3). However, juveniles of *G. salinus* hatched and survived in a broader salinity range than those of *P. maeoticus*

and *G. tigrinus* (Figs. 3 and 4). *Gammarus salinus* hatched at salinities from 1 to 34 g/Kg, *P. maeoticus* from 0 to 22 g/Kg, and *G. tigrinus* from 4 to 32 g/Kg. The mean number of hatched juveniles was also the highest in the case of *G. salinus*, with 39.0, 19.2 and 15.7 juveniles in the control, low and high salinity treatment, respectively. The mean numbers of hatched juveniles of *P. maeoticus* were 5.0, 4.8 and 3.8, while those of *G. tigrinus* were 17.9, 18.3 and 13.5, respectively. In the third week of the experiment, all juveniles of *G. salinus* died at 1 and 7 g/Kg, while in the case of *P. maeoticus* and *G. tigrinus*, there was no survival at 0, 1, 19 and 23 g/Kg and 5, 27 and 31 g/Kg, respectively (Fig. 3). Consequently, until the end of the experiment juveniles of *G. salinus* survived in a slightly narrower salinity range than their parents (i.e., adults and juveniles survived in salinities from 0 to 40 g/Kg and 3 to 33 g/Kg, respectively; Fig. 4). The mean mortalities across all salinities were 44.2%, 76.8% and 43.5% in the control, low and high salinity treatment, respectively. Statistical analyses determined a significant difference among the treatments for juveniles of *G. salinus* (Kruskal-Wallis test, $\chi^2 = 12.189$, df = 2, p-value = 0.0022), with a significantly higher mortality in the low salinity treatment when compared to the control (Wilcoxon rank sum test, p = 0.004). The difference was not found between the low and high salinity treatments due to low statistical power (Wilcoxon rank sum test, p = 0.2322). In the case of *P. maeoticus* and *G. tigrinus*, juveniles survived in much narrower salinity ranges than their parents (i.e., adults and juveniles of *P. maeoticus* survived in salinities from 0 to 34 g/Kg and 5 to 23 g/Kg, respectively, while those of *G. tigrinus* from 0 to 40 g/Kg and 9 to 23 g/Kg, respectively; Fig. 4). The mean mortalities of juveniles of *P. maeoticus* were 73.4%, 60.0% and 92.0% in the control, low and high salinity treatment, respectively; those of *G. tigrinus* were 52.3%, 63.3% and 72.2%, respectively. There was no significant difference in mortality of juveniles among

treatments neither for *P. maeoticus* nor *G. tigrinus* (Kruskal-Wallis test, $\chi^2 = 5.459$, df = 2, p-value = 0.0653 and $\chi^2 = 4.042$, df = 2, p-value = 0.1325, respectively).

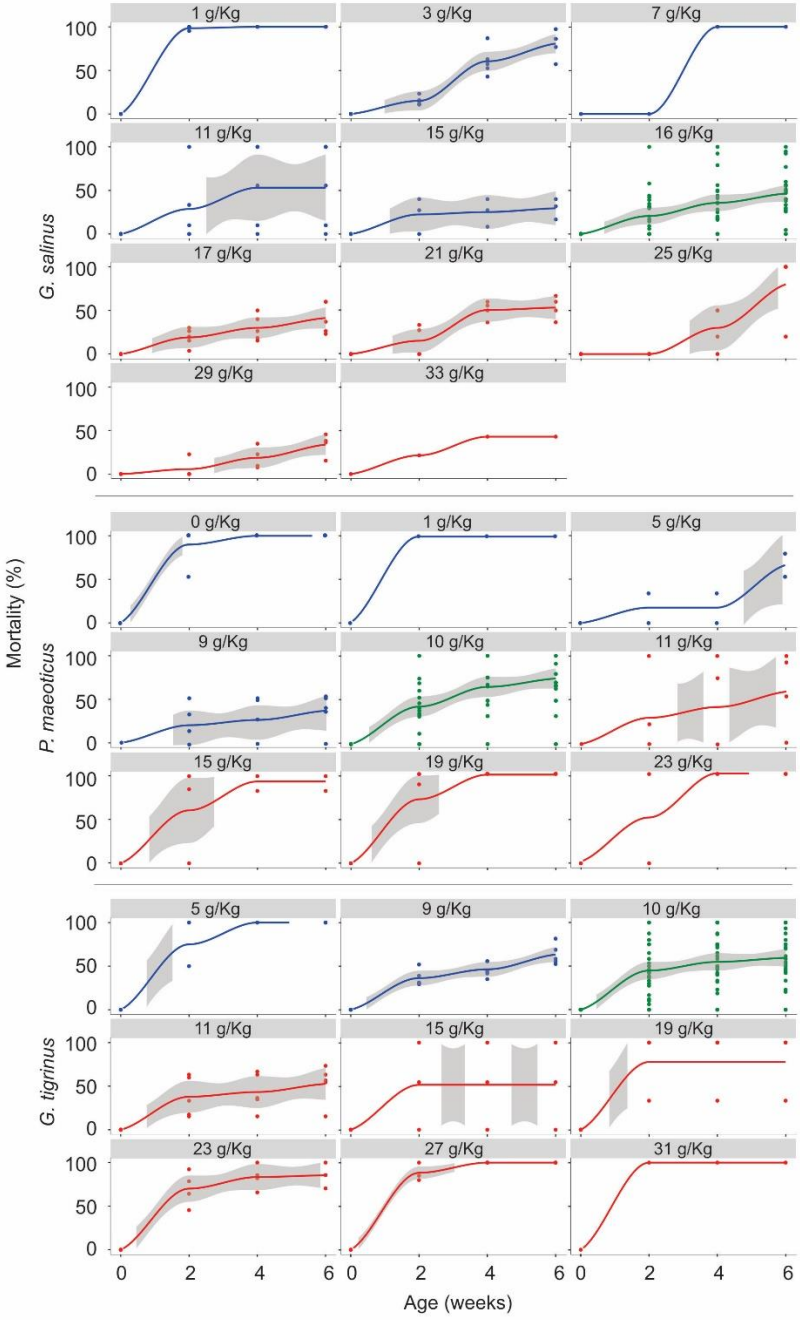

Fig. 3. Mortality of juveniles of *G. salinus*, *P. maeoticus* and *G. tigrinus*, with each panel representing the respective hatching and rearing salinity of the juvenile cohort. Experimental treatments are depicted in green, blue and red for control, low and high salinity treatment, respectively. Respective 95% confidence intervals are presented by the gray area.

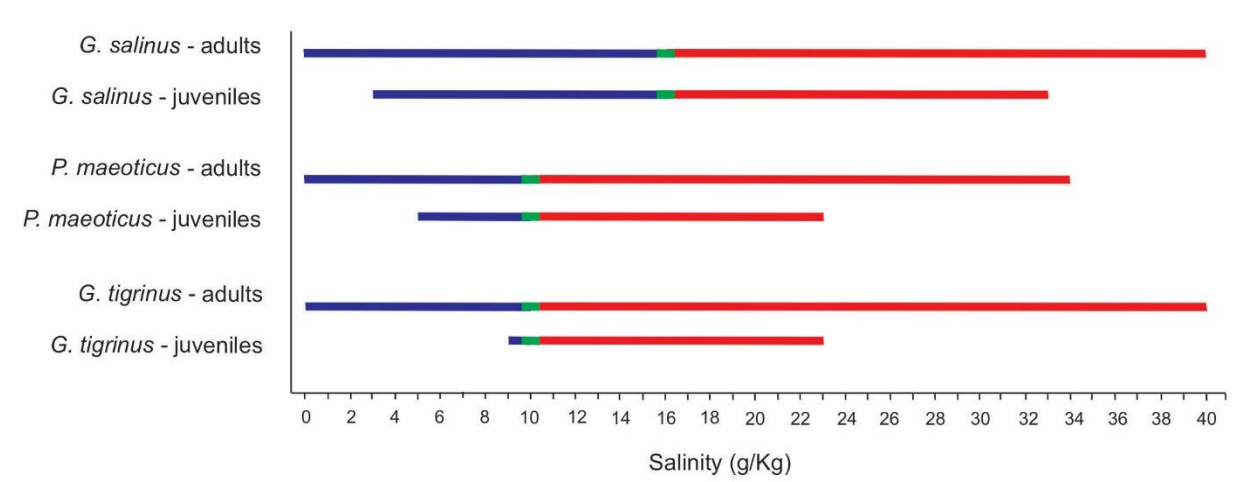

Fig. 4. Salinity tolerance of adults and juveniles (i.e., salinity range at which tested individuals survived until the end of experiment) of *G. salinus*, *P. maeoticus* and *G. tigrinus*. Salinity tolerance was based on any number of individuals surviving a particular salinity instead of LD50 due to a low number of individuals tested per salinity. Experimental treatments are depicted in green, blue and red for control, low and high salinity treatment, respectively.

Growth rates of juveniles

Growth of juveniles differed among species and treatments, with the fastest growth recorded for juveniles of *G. salinus* in the high salinity treatment at 25 g/Kg, and the slowest for *P. maeoticus* in the control and high salinity treatment at 10 and 11 g/Kg, respectively (Fig. 5). The mean cephalon length of *G. salinus* increased from 375.8 (week 0) to 907.7 µm (week 6), with the juveniles at 3, 16 and 29 g/Kg having a significantly shorter length than those at the other salinities (Table 4; Fig.5). In the case of *P. maeoticus*, the mean cephalon length increased from 345.1 (week 0) to 503.3 µm (week 6, Fig. 5). There was no significant difference in cephalon length among the different salinities (ANOVA, $F(1, 51) = 0.503$, $p = 0.481$). Finally, the mean cephalon length of juveniles of *G. tigrinus* increased from 348.2 (week 0) to 830.3 µm (week 6), with juveniles hatched at 23 g/Kg having significantly shorter cephalons than those hatched at 9, 10, 11 and 15 g/Kg ($F(5, 190) = 6.075$, $p = < 0.0001$; Table 5; Fig. 5).

Table 4 Pairwise comparisons of the effect of salinity on the cephalon growth of *G. salinus* in week 6.

		Hatching salinity of the juvenile cohort (g/Kg)							
		3	**11**	**15**	**16**	**17**	**21**	**25**	**29**
Hatching salinity of the juvenile cohort (g/Kg)	11	**<0.0001**	-	-	-	-	-	-	-
	15	**<0.0001**	1.0000	-	-	-	-	-	-
	16	1.0000	**0.0066**	**<0.0001**	-	-	-	-	-
	17	**<0.0001**	1.0000	1.0000	**<0.0001**	-	-	-	-
	21	**<0.0001**	1.0000	1.0000	**0.0010**	1.0000	-	-	-
	25	**0.0015**	1.0000	1.0000	0.2541	1.0000	1.0000	-	-
	29	0.4129	**0.0384**	**<0.0001**	1.0000	**0.0046**	**0.0419**	**0.0180**	-
	33	**0.0371**	1.0000	**0.0390**	1.0000	1.0000	1.0000	0.2909	1.0000

Note: Wilcoxon rank sum test with Bonferroni adjustment was used to test the effect among different salinities.

Table 5 Pairwise comparisons of the effect of salinity on the cephalon growth of *G. tigrinus* in week 6.

		Hatching salinity of the juvenile cohort (g/Kg)				
		9	**10**	**11**	**15**	**19**
Hatching salinity of the juvenile cohort (g/Kg)	10	0.8778	-	-	-	-
	11	1.0000	1.0000	-	-	-
	15	0.8032	1.0000	1.0000	-	-
	19	0.5443	1.0000	1.0000	1.0000	-
	23	**0.0252**	**<0.0001**	**0.0074**	**0.0332**	0.1428

Note: Tukey HSD test was used to test the effect among different salinities.

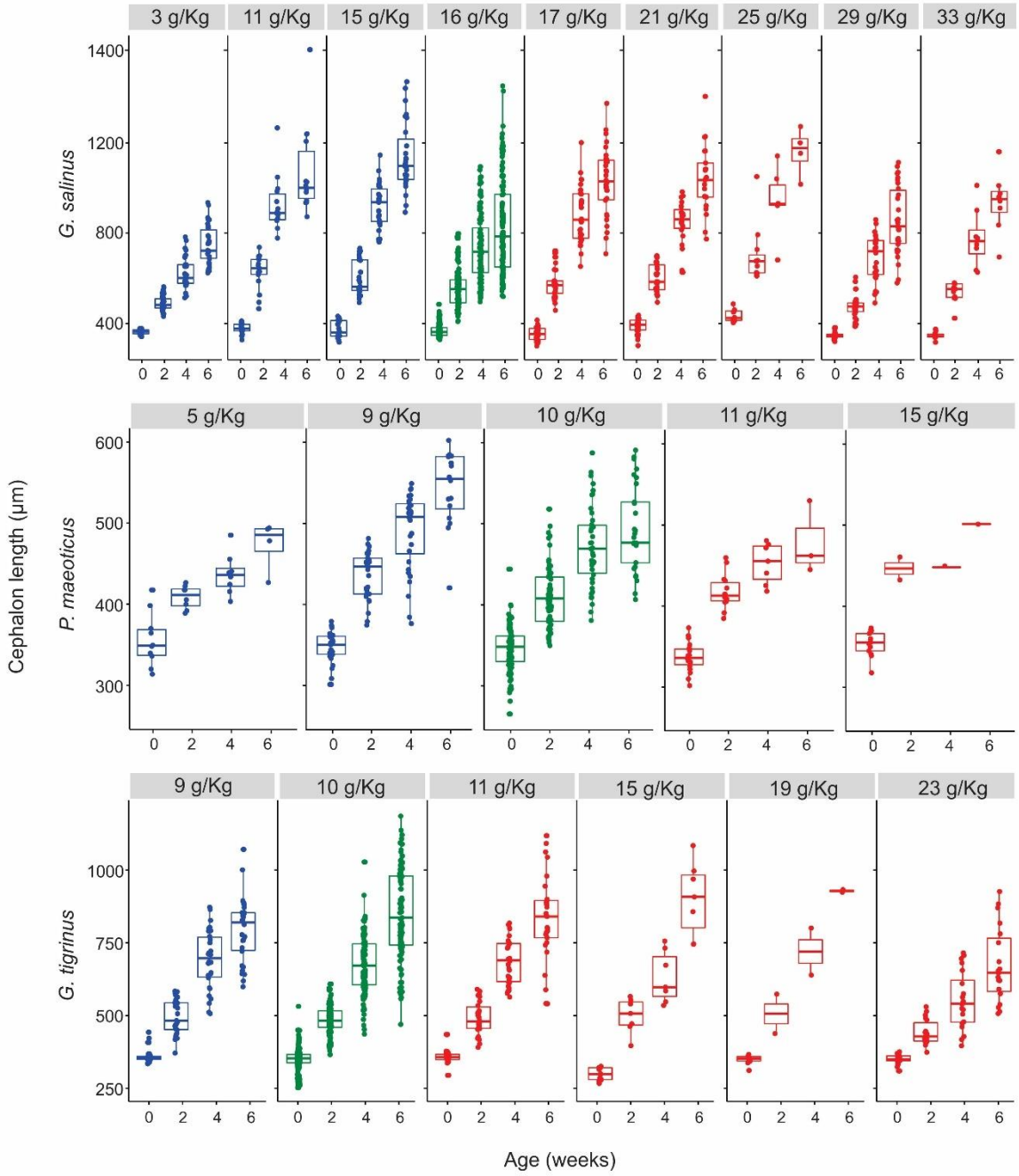

Fig. 5. Cephalon length (µm) of juveniles of *G. salinus*, *P. maeoticus* and *G. tigrinus*, with each panel representing the respective hatching and rearing salinity of the juvenile cohort. Experimental treatments are depicted in green, blue and red for control, low and high salinity treatment, respectively. The boxes represent upper and lower quartiles with median line, while dots represent individual measurements.

Discussion

Due to an increasing number of NIS worldwide and their impacts on ecosystems and biodiversity (Seebens et al., 2018; Simberloff, 2011; Strayer et al., 2006), recently numerous studies have been testing species resilience to changes in environmental conditions, such as temperature and salinity (Casties, Clemmesen, & Briski, 2019; McFarland et al., 2015; Paiva et al., 2018). However, many of those studies were conducted on adult stages, while it still remains unclear whether those species will reproduce in and how their juveniles would respond to those changing conditions. In this study, we compared the salinity tolerance of adults and juveniles of three gammarid species originating from Northern Europe, the Ponto-Caspian region and North America to determine whether juveniles would perform equally well as adults. Additionally, we compared our study with Paiva et al. (2018). Not surprisingly, our study determined that both adults and juveniles of all three species tolerated wide ranges of salinity, with juveniles of *G. salinus* tolerating only slightly narrower salinity range than their parents, while those of *P. maeoticus* and *G. tigrinus* much narrower range. At the end of the experiments, mortalities of adults of *G. salinus* and *P. maeoticus* were significantly different in the high salinity treatment, but not in the low, with *P. maeoticus* having 100% mortality in the high salinity treatment above 34 g/Kg. Importantly, our study determined better performance of juveniles of *G. salinus* in higher salinities and those of *P. maeoticus* in lower salinities. Consequently, even though the adults in our study did not reveal exactly the same pattern of salinity tolerance as determined by Paiva et al. (2018), we found similar pattern for juveniles. Based on juvenile salinity tolerance, our study supports further the finding of Paiva et al. (2018) that Northern European species perform better in higher, while Ponto-Caspian in lower salinities.

By investigating the salinity tolerance of adult euryhaline gammarids, Paiva et al. (2018) determined different patterns of tolerance among species from different regions, with Northern European taxa showing lower mortality in fully marine and Ponto-Caspian taxa in freshwater conditions. Therefore, the authors suggested that Northern European species and Ponto-Caspian species may be of marine and freshwater origin, respectively. Interestingly, even though we tested the same populations of *G. salinus* and *P. maeoticus* as did Paiva et al. (2018), we found similar pattern of salinity tolerance only for juveniles, but not for adults. In addition, we have to emphasize that even though juveniles of *P. maeoticus* in our study performed better in lower salinities, they did not survive in freshwater conditions. There may be two reasons why juveniles failed to survive in fresh water. The first and most probable reason was very low number of hatched juveniles. As mortality of juveniles in *r*-strategy species is very high (Ramírez-Llodra, 2002), the mortality in freshwater conditions in our experiments may be simply due to chance, not to environmental conditions. The second reason may be low genetic diversity of our population, as the population was started with 96 individuals, transferred to the laboratory, and kept for one and a half years before the experiments were conducted. However, a more pronounced difference between our study and that of Paiva et al. (2018) was in the survival of adults, with our study not demonstrating better survival of *G. salinus* and *P. maeoticus* in fully marine and freshwater conditions, respectively, as Paiva et al. (2018) determined. Though, the lower salinity tolerance of adults in our study may be caused by the use of pairs in precopula rather than single individuals as in Paiva et al. (2018). Gammarid females can be fertilized only for a short period of time after moulting. Therefore, a male finds a pre-moult female and they form a precopula pair, with the male carrying the female (Hynes, 1955; Jormalainen, 1998; Parker, 1974). The precopula stage brings a number of costs to both sexes, such as energetic costs of moulting and prolonged mate guarding to a female

and those of locomotion of the pair to a male (Elwood & Dick, 1990; Jormalainen, 1998; Sparkes, Keogh, & Pary, 1996). Finally, an additional reason for better survival of *P. maeoticus* in fresh water in Paiva et al. (2018) than in our study may be connected to water chemistry as the experiments in Paiva et al. (2018) were conducted using the ambient water of the species collection site, while our experiments were conducted using Baltic Sea water and tap water in Germany. Consequently, as our precopula pairs were exposed not only to salinity stress of our experiments, but also to reproductive stress, and in the case of *P. maeoticus* possible differences in water chemistry used in the experiments, the energetic costs of the species were probably exceeded leading to lower survival than that in Paiva et al. (2018).

Hatching success of the three species differed among species and in the case of *G. salinus* among treatments. In the control treatments, the hatching success of *G. salinus* was eight-fold higher than that of *P. maeoticus*, and two-fold higher than that of *G. tigrinus*. Interestingly, while the numbers of hatched juveniles of *G. salinus* in the low and high salinity treatments were half of that in the control this was not the case for *P. maeoticus* and *G. tigrinus*. The differences in hatching success among species may be related to their different reproductive strategies. *Gammarus salinus* produces three to seven broods per generation with approximately 30 juveniles per brood, *P. maeoticus* only three broods with approximately nine juveniles per brood, while *G. tigrinus* produces at least ten broods with ten to 50 juveniles per brood (Nazarhaghighi, Shabanipour, Zarghami, & Etemadi-Deylami, 2013; Sutcliffe, 1993 and references therein). Furthermore, in our study juveniles of *G. salinus* and *G. tigrinus* hatched in much wider ranges of salinities than those of *P. maeoticus*. Environmental conditions often largely affect hatching success and development (Donelson, Munday, & McCormick, 2009; English, Pen, Shea, & Uller, 2015). Often, environmental stress causes parents to use their energy resources for their own

survival instead of for the reproduction of offspring (Glazier, 1999). Consequently, viability of embryos and number of broods produced may be lower, and hatched juveniles smaller and weaker (e.g. Mills & Fish, 1980, Neuparth et al., 2002; Steele & Steele, 1991; Vlasblom & Bolier, 1971). Therefore, in our study, besides the different reproductive strategies among species, salinity stress resulted in the production of smaller broods of *G. salinus* in the low and high salinity treatments, and even prevented hatching of *P. maeoticus* in salinities above 23 g/Kg.

Juvenile survival and growth also differed among the species and particularly among the treatments. While juveniles of *G. salinus* and *G. tigrinus* had better survival in higher salinities, *P. maeoticus* survived better in lower. Furthermore, the growth of juveniles of *G. salinus* was slower in very high and low salinities, while juveniles of *P. maeoticus* and *G. tigrinus* did not survive at all in those salinities. These results suggest that stressful environmental conditions affect the use of energy resources of juveniles, as they redirect energy from growth to survival (Anger, Spivak, & Luppi, 1998; Torres, Giménez, & Anger, 2011). In addition, smaller and weaker juveniles, often produced by stressed parents, commonly will grow slower or even run out of energy due to reduced starting resources and additional environmental stress (e.g., Mills & Fish, 1980, Neuparth et al., 2002; Steele & Steele, 1991; Vlasblom & Bolier, 1971). In our study, *P. maeoticus* adults were probably experiencing great stress as salinity was increasing, and consequently, though they survived until 34 g/Kg, they did not produce any juveniles above 23 g/Kg. In contrast, *G. salinus* experienced a gradual increase in stress as salinity went further from the species ambient salinity, with freshwater conditions being too stressful for production of juveniles. Interestingly, even though *G. tigrinus* adults experienced great stress and high mortality, they did not seem to have redirected much energy from reproduction to their own survival, and they were consequently able to produce juveniles in almost the whole range of salinities they survived in. Thus, our study

supports the finding of Paiva et al. (2018), where *G. salinus* performs better in higher, while *P. maeoticus* in lower salinities.

In our study, as well as in Paiva et al. (2018) and Casties et al. (2019), the mortalities of adults of *G. tigrinus* were the highest in the control treatment, irrespective of whether salinity or temperature tolerance was examined. Interestingly, we did not observe the same pattern for juveniles. Juveniles in our study had the lowest mortality in the control treatment when compared to those in the low and high treatments, as well as to the mortality of their parents in any treatment. Both Paiva et al. (2018) and Casties et al. (2019) suggested that dark spots, regularly observed on animals, were most likely parasitic oomycetes that reduced immune function of animals (Kestrup, Thomas, van Rensburg, Ricciardi, & Duffy, 2011), and as the parasite was not able to tolerate changes in environmental conditions of the experiments, the highest mortalities were observed in the control treatments. In our study, dark spots were also observed on individuals. However, our juveniles did not demonstrate low performance in the control treatment. Therefore, either the oomycete cannot be transferred directly from parents to offspring or an additional parasite that needs an additional host might be reducing the immune system of adults, such as microphallid trematodes (MacNeil et al., 2003; Mouritsen, Tompkins, & Poulin, 2005; Prugnolle, Liu, de Meeûs, & Balloux, 2005). Consequently, when examining environmental tolerance of species, the possibility of parasitic infections and/or other diseases of the tested populations should be taken into account.

Conclusion

Global biodiversity and ecosystems are highly impacted by anthropogenic activities, such as climate change and introduction of NIS (Capinha et al., 2015; IPCC, 2014; Sala et al., 2000). Changes in ecosystems due to increasing temperature, heatwaves, acidification and decreasing

salinities, pose additional energetic costs to native species, with some of them hardly coping with these stressors (Chapman, 2017; IPCC, 2014; Solan & Whiteley, 2016). In addition, continuously arriving NIS, which are often more resistant to multiple stressors and preadapted to anthropogenic impact, use these opportunities putting an additional burden on already stressed ecosystems (Holopainen et al., 2016; Hufbauer et al., 2011; IPCC, 2014). Consequently, numerous studies have been testing species resilience to environmental fluctuations, yet, rarely both adults and juveniles were tested (Casties et al. 2019; McFarland et al., 2015; Paiva et al. 2018). By comparing salinity tolerance of adults and juveniles of three gammarid species originating from Northern Europe, the Ponto-Caspian region and North America, our study demonstrated that juveniles were not able to tolerate the same stress as adults. Furthermore, geographic origin of species plays an important role in their environmental tolerance. Even though our tested species came from similar ambient salinities, our study determined significant differences in direction of salinity tolerance, with Northern European species performing better in higher, while Ponto-Caspian in lower salinities. Here, we emphasize that additional studies are needed to confirm if these findings can be generalized. For example, *P. maeoticus*, tested here, has invading history only at two locations close to the Ponto-Caspian region (Fig. 1). Therefore, it would be beneficial to determine if *P. robustoides*, which is a widespread NIS (Bij de Vaate et al., 2002), has the same salinity tolerance or if it is even more resistant to salinity stress than *P. maeoticus*. Likewise, populations of species in invaded areas may differ from the ones in native regions. Here, we tested the population of *G. tigrinus* from a non-indigenous region, where low genetic diversity due to the founder effect or high genetic diversity due to introgression from diverse source populations may have skewed mean fitness of our tested population. Therefore, studies testing populations from both native and non-indigenous regions would provide valuable information in determining stress tolerance of diverse

taxa. Finally, we emphasize that multiple factors, such as early life-history stages, condition of the tested populations, as well as water chemistry and parasitism, should be taken into account in determining environmental tolerance of species and in constructing models to predict changes in species distributions, resilience of ecosystems and biodiversity change.

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Data Availability Statement

The primary dataset containing experimental results is available at Pangaea:

https://doi.org/10.1594/PANGA EA.90829, and Dryad: https://doi.org/10.5061/dryad.3n5tb 2rf5.

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Chapter 4

Consistency of aquatic enclosed experiments: the importance of scale and ecological complexity

Submitted in Diversity and Distributions

Filipa Paiva [1, 2], Dennis Brennecke [3, 4, 5], Christian Pansch [6] and Elizabeta Briski [1]

[1]GEOMAR, Helmholtz-Zentrum für Ozeanforschung Kiel, Düsternbrooker Weg 20, 24105 Kiel, Germany

[2] MARE – Marine and Environmental Sciences Centre, Agência Regional para o Desenvolvimento da Investigação Tecnologia e Inovação (ARDITI), Edifício Madeira Tecnopolo, Caminho da Penteada, 9020-105 Funchal, Madeira, Portugal

[3] Institute for Terrestrial and Aquatic Wildlife Research, University of Veterinary Medicine Hannover, Foundation, Werftstr. 6, 25761 Büsum, Germany

[4] Department of Biology, Marine Biological Research Centre, University of Southern Denmark, Hindsholmvej 11, 5300 Kerteminde, Denmark

[5] Leibniz Institute for Science and Mathematics Education, Olshausenstraße 62, 24118 Kiel, Germany

[6] Department of Environmental & Marine Biology, Åbo Akademi University, Turku, Finland

Abstract

Aim: Marine and freshwater ecosystems are increasingly threatened by human activities. For over a century, scientists have been testing many biological, chemical and physical questions to understand various ecosystems and their resilience to different stressors. While the majority of experiments were conducted at small-scale laboratory settings, lately large mesocosm experiments have become more and more common. Yet, it still remains unclear how the scale (i.e. space) and ecological complexity (i.e. community vs. limited number of species) of experiments affect the results and to what extent different experimental types are comparable.

Innovation: Here, we conducted two types of experiments, run at different scale and ecological-complexity levels (i.e. outdoor large-scale community-level mesocosm vs. indoor small-scale two-species laboratory experiment), to assess the effects of marine heatwaves on two gammarid species.

Main Conclusions: Our approach detected differences in abundance and relative population growth between the two experimental types for one out of the two tested species, but no difference in heatwave impacts on any of the species, independently of which experimental type was used. The larger space in the mesocosm, accompanied with inclusion of the community, benefited this species, demonstrating stronger performance in the mesocosm than in the laboratory experiment. Though, our study design cannot directly distinguish if scale or ecological complexity of the experiments, or both, caused the observed discrepancy in our findings. Furthermore, inconsistency in results among laboratory experiments complicates the extrapolations and generalization of the laboratory results. Yet, our findings indicate the importance of space, density-dependent effects, biotic interactions and complexity of natural environments in buffering, or boosting, the direct effects of environmental stress on organisms. Therefore, we urge the use of large-scale

community-level mesocosm experiments instead of small-scale single-species laboratory ones whenever possible, and emphasize a necessity of great caution when interpreting the results of laboratory experiments.

Keywords: gammarids, heatwaves, mesocosm, laboratory experiment, large-scale experiments, small-scale experiment

Introduction

The speed and extent of current changes in the Earth's climate and environments have reached unprecedented rates, being faster than any previously observed (IPBES, 2019; Stillman, 2019). Over the last several decades, marine and freshwater ecosystems have been significantly affected by rising temperatures and other anthropogenic activities, such as increased nutrient flows, pollution, overexploitation and introduction of non-native species (Capinha, Essl, Seebens, Moser & Pereira 2015; Chapman, 2017). Consequently, scientists have been using field observations and experiments, often in combination with mathematical modeling, to understand coastal ecosystems and their resilience to stress (Petersen, Denninson, Kennedy & Kemp, 2009). Through time, due to development in science and technology, new and more advanced approaches and methods have been designed and developed, including valuable improvements in experimental set-ups, data analyses and computation (Evans, 2012; Woodward, Perkins & Brown, 2010).

While some ecological research can be successfully conducted by observational studies with specific support of mathematical modelling, others need manipulative experiments to prove or refute the tested hypotheses (Stewart et al., 2013; Widdicombe, Dupont & Thorndyke, 2010). There is no standard experimental design that fits all research questions, and the approaches used may differ not only in spatial scale, but also in ecological complexity. While spatial scale is defined by variables such as length, area and volume, ecological complexity is characterized by species

diversity and levels of ecological organization (Petersen et al., 2009). In this context, research approaches can vary tremendously. On the one side, there are laboratory experiments which are usually conducted at small-scale and individual or single-species levels. These experiments often test many of the basic biological, chemical, and physical questions in a controlled environment, allowing for high replication (Widdicombe et al., 2010). However, they are often simpler in terms of complexity and exclude important ecological and biological components present under natural settings. As they create an artificial environment, their validity often raises concern among the scientific community (Carpenter, 1996; Widdicombe et al., 2010; Stewart et al., 2013). On the other side, large-scale mesocosm experimental infrastructure and approaches have been significantly improved in recent years, allowing replication of "near-realistic" scenarios including important variables occurring in the natural environments by using subsets of natural ecosystems (e.g. Dzialowski et al., 2014; Kraufvelin et al., 2006, 2010, 2020; Pansch & Hiebenthal, 2019; Wahl et al., 2015). By creating almost natural ecological and biological dynamics of ecosystems while the tested variables are manipulated and controlled, the results and conclusions deduced from mesocosm experiments are usually assumed to be more reliable and predictive than laboratory tests (Kraufvelin et al., 2006, 2010, 2020; Petersen, Cornwell & Kemp, 1999; Widdicombe et al., 2010). However, it should be emphasized that though mesocosm experiments use a subsample of a natural ecosystem, they are still a simplification of nature, and this should be considered when drawing wider conclusions from the observed results and when forecasting future scenarios (Petersen et al., 2009; Widdicombe et al., 2010).

Extreme isolated events associated with global warming, namely heatwaves, have lately raised the awareness of the scientific community due to their increasing frequency worldwide in both marine and freshwater ecosystems (Holbrook et al., 2019; Huber, Wagner, Gerten & Adrian,

2012; Oliver et al., 2018). Heatwaves are warm isolated events that last for five or more days at temperatures warmer than the 90th percentile based on a 30-year historical baseline period (Hobday et al., 2016). There are summer and winter heatwaves, with many of the former having devastating impacts on ecosystems, while some of the latter being even beneficial (Cavole et al., 2016; Hobday et al., 2016). In 2003, in the Northwestern Mediterranean region, one of the first documented impacts of a heatwave occurred, causing extensive mortality among numerous benthic communities (Garrabou et al., 2009). Since then, several studies have reported similar events worldwide, such as the Western Australia heatwave in 2011 (Pearce & Feng, 2013) and the Northwest Atlantic heatwave only one year later (Mills et al., 2013). Field observations have determined strong responses of marine and freshwater environments to summer heatwaves, such as toxic cyanobacteria blooms (Joehnk et al., 2008), mass coral bleaching (Hughes et al., 2017) and extensive mortalities of important commercial fish species (Caputi et al., 2016). As these isolated climatic events can negatively affect aquatic communities, and it is expected that they will increase in their severity and frequency (Cavole et al., 2016; Smale et al., 2019), it is of great importance to understand the responses and resilience of ecosystems to this climatic abnormality to be able to better protect coastal habitats (Frölicher, Fischer & Gruber, 2018; Sorte, Williams & Carlton, 2010).

Although a great number of studies has been conducted to determine the responses of single-species or communities to global warming and other anthropogenic impacts using mesocosm and laboratory experiments (e.g., Casties, Clemmensen & Briski, 2019; Madeira, Leal, Diniz, Cabral & Vinagre, 2018; Pansch et al., 2018; Wahl et al., 2020), it still remains unclear how the type of the experiment (i.e. scale and ecological complexity) affects the outcome and to what extent the two types of experiments are comparable. In this study, we conducted two experiments

using different scale and ecological-complexity levels: *i*) an outdoor large-scale community-level mesocosm and *ii*) a small-scale two-species laboratory experiment, to assess the effects of heatwaves on two gammarid species from the Baltic Sea. To be able to compare the results of the two types of experiments, after three months of rearing animals in different set-ups, relative population growth was calculated for each species and for each experimental type. We tested the null hypotheses assuming no difference in population growth: *i*) for any of the species between the two experimental types; *ii*) between the two species in each experimental type; and *iii*) among different treatments.

Methods

Specimen collection

Two species from the superfamily Gammaroidea (i.e. *Gammarus locusta* and *G. salinus*) were collected in April and May 2015 for the mesocosm and in April and May 2016 for the laboratory experiment. *Gammarus locusta* was collected in Falckenstein, Germany (54°40' N 10°20' E), while *G. salinus* was collected in Kiel, Germany (54°33' N 10°15' E), two sampling locations only a few km apart (<10). Specimens were transported in their ambient water to the laboratories at GEOMAR in Kiel, where each individual was morphologically identified according to Köhn & Gosselck (1989). Additional species for the mesocosm experiment were collected within the Kiel Fjord between May 4 and 6, 2015 (i.e. *Zostera marina*, *Fucus vesiculosus*, *Cerastoderma edule*, *Idotea balthica*, *Littorina littorea*, and *Mytilus edulis*; for details see Pansch et al., 2018).

Experimental set-up

To determine if the type of the experiment affects the results of the experiment, we have conducted two experiments using different scale and ecological-complexity levels: *i*) an outdoor

large-scale community-level mesocosm experiment – hereafter referred as mesocosm experiment; and *ii*) a small-scale two-species laboratory experiment – hereafter referred as laboratory experiment, to assess the effects of heatwaves on *G. locusta* and *G. salinus*. The mesocosm experiment was conducted from May 6th until August 20th, 2015, and the laboratory experiment from May 17th until September 2nd, 2016. The experimental set up of the mesocosm experiment consisted of six thermally insulated large mesocosms, placed outdoors, with each being divided into two independent experimental tanks of 1500 L (Figure 1a, c). Water has been completely exchanged daily by a constant flow-through of 1800 L per day of unfiltered seawater allowing for natural fluctuations in both abiotic and biotic conditions (Wahl et al., 2015). Only temperature was manipulated (see below; Figure 2). Forty-eight individuals of each *G. locusta* and *G. salinus* were added to each experimental tank, together with two main habitat-forming macrophytes of Western Baltic Sea: *Z. marina* and *F. vesiculosus,* and their most abundant associated fauna: *C. edule*, *I. balthica*, *L. littorea*, and *M. edulis* (for details see Pansch et al., 2018). The experimental set up of the laboratory experiment consisted of six water baths (52 L each), with two experimental tanks (13.5 L each) set inside each bath (Figure 1b, d). Temperature of water in the experimental tanks was manipulated by regulating water temperature of the water baths, following the same pattern as in the mesocosm experiment (see below; Figure 2). Here, we acknowledge that this two by two block design may have some effect on our results, but it was necessary for temperature regulation. Water in the experimental tanks has been completely exchanged approximately every hour by a constant flow-through of filtered seawater (20 µm). Twenty individuals of each *G. locusta* and *G. salinus* were added to each experimental tank. We emphasize here that there was a large difference between the density of gammarids per volume of water introduced into the two experimental scales

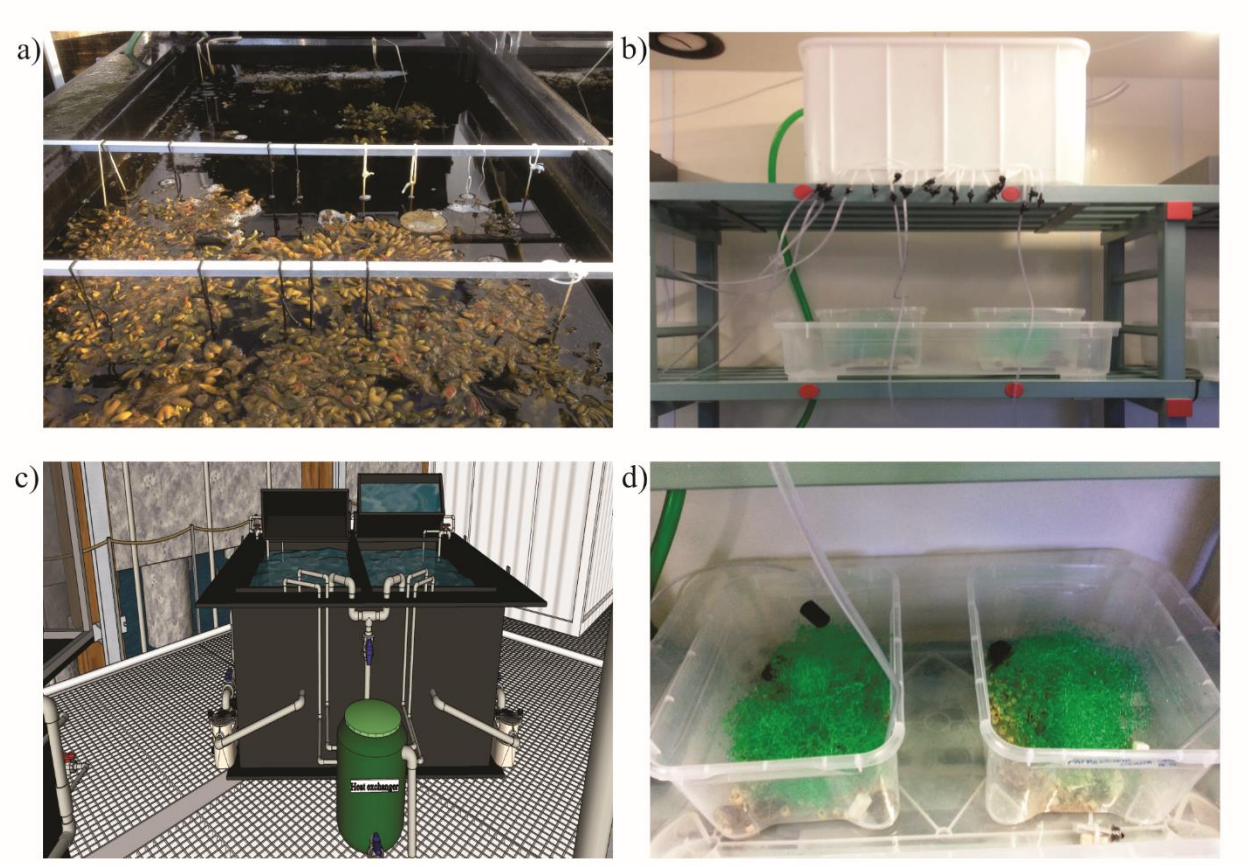

Fig. 1. Overview of the two types of experimental set-up (a) mesocosm tank with tested community from above (see Pansch et al., 2018 and Wahl et al., 2015), (b) laboratory setting including the main water storage tank with water distribution hoses leading to the experimental tanks, (c) a scheme of the mesocosm tanks (Wahl et al., 2015), and (d) laboratory tanks with animals and artificial refugia structures from above.

(i.e. the mesocosm and laboratory experiments), but this high density of individuals in the laboratory scale was necessary to secure enough healthy individuals of different sex to start the populations. From our previous experience with raising the tested species in the laboratory conditions, we are confident that the density of individuals in the laboratory experiment was not too high to cause density-dependent effects. To improvise the natural environment to a certain degree, sand, stones, empty mussel shells and artificial mesh were added to tanks, while animals were fed *ad libitum* with *F. vesiculosus* and a mixture of commercial food flakes for crustaceans (Tetra Mix). During the experiments, the experimental tanks were covered by lids and

continuously aerated. Over the course of both experiments (i.e. the mesocosm and the laboratory experiments), there was limited or no contact with the animals. At the end of the experiments, individuals were collected by a sieve (mesh size of 335µm), stored in ethanol and later morphologically identified.

Heatwave treatments

The experimental design consisted of three treatments: i) control; ii) one heatwave; and iii) three heatwaves. Each treatment consisted of four replicates. The treatments were designed based on a statistical analysis of 15 years of daily mean temperature records from the experimental site. Using a generalized additive mixed model (GAMM), daily mean temperatures from May to September for the control treatment were designed (for details see Pansch et al., 2018; Figure 2). In the one heatwave treatment, the heatwave started on 90th day of the experiments, while in the three heatwaves treatment, the heatwaves started on 25th, 56th, and 90th day, respectively. Each heatwave lasted nine days, where during the first three days temperature was increasing, reaching a peak phase on the fourth day and staying for four days at the peak phase, followed by two days of cooling. The peak phase of the first two heatwaves was 3.6°C, while that of the third one was 5.2°C higher than the control treatment (for details see Pansch et al., 2018). For more details on the heating system of the mesocosm experiment, see Wahl et al. (2015). In the case of the laboratory experiment, temperature inside the experimental tanks was daily manually adjusted using aquaria heaters submerged into the water baths (Aqua Medic titanium heaters 100W), with submersed pumps insuring homogenous mixing of water inside the baths (Figures 1 and 2). The temperature inside each water bath was monitored every 30 minutes throughout the experiment using temperature loggers (HOBO Pendant® Temperature Data Logger).

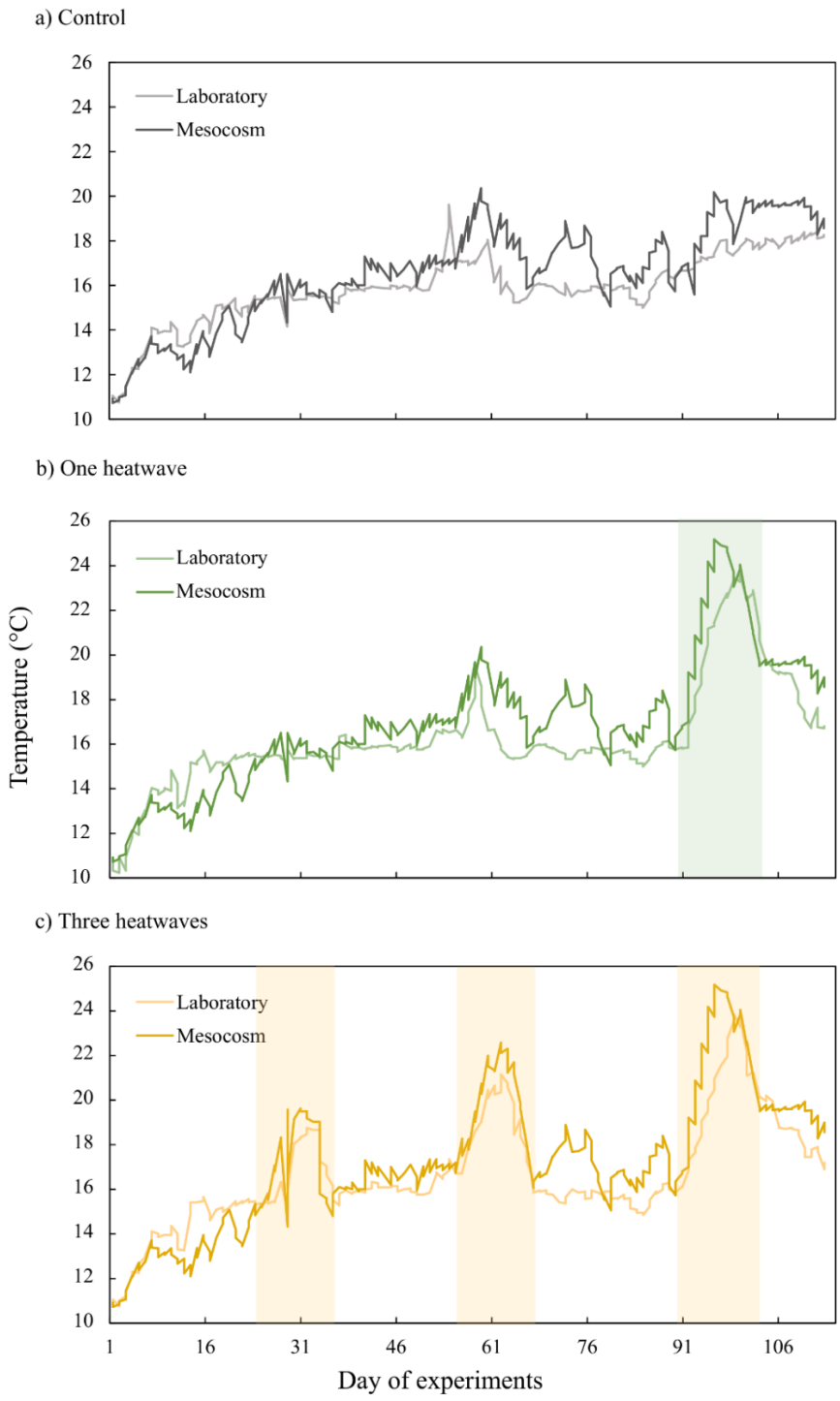

Fig. 2. Implemented temperature profiles in mesocosm and laboratory experiments. Temperatures are provided from May 6th to August 20th 2015 for mesocosm and from May 17th to September 2nd 2016 for laboratory experiment for (a) the control, (b) one heatwave, and (c) three heatwaves treatment. Heatwaves periods are highlighted by green and yellow rectangles for the one and three heatwaves treatment, respectively.

Statistical Analysis

First, we calculated the percentage of population growth for each replicate of each treatment in each experimental type following the equation:

$$R = 100 * k \quad (1)$$

where R is the percent change per unit time (i.e. growth rate) and k is the fractional change per unit time (Bartlett 1993). The k was calculated as follows:

$$k = (N_{end} - N_{beg}) / N_{beg} \quad (2)$$

with N_{end} being the number of individuals at the end of the experiment and N_{beg} being the number of individuals at the beginning of the experiment. Then, variation in percentage of population growth between the species within experimental type was compared using two-way factorial analysis of variance (ANOVA) where percentage of population growth was the dependent variable, and species (i.e. *G. locusta* and *G. salinus*) and heatwave treatments (i.e. control, one heatwave and three heatwaves) were the independent variables. Two separate ANOVAs were conducted, each for one type of experiment. For all comparisons, the assumptions of normality and homogeneity of variances were confirmed using the Shapiro-Wilk‘s W-Test and Fligner-Killeen Test, respectively. Statistical analyses were performed using the R software, version 3.5.2 (R Core Team, 2018). Data visualization was conducted by “ggpubr” and “ggplot2” packages in R (Kassambara, 2018; Wickham, 2016).

Results

In the mesocosm experiment, there was no significant difference in the percentage of population growth between the two species (ANOVA, $F(1,18) = 3.563$, $p = 0.075$), though *G. locusta* had slightly higher mean number of individuals at the end of the experiment in all

treatments when compared to *G. salinus* (i.e. mean and standard deviation were 485.3 ± 162.7, 664.5 ± 162.8 and 560.5 ± 353.1 for *G. locusta*, and 336.0 ± 244.8, 427.0 ± 144.9 and 451.3 ± 132.8 for *G. salinus* in the control, one heatwave and three heatwaves treatment, respectively; Table 1). In contrast, in the laboratory experiment, the percentage of population growth between the two species was significantly different (ANOVA, $F(1,18) = 23.499$, $p < 0.001$), with *G. salinus* having significantly higher relative population growth across all treatments. While number of individuals of *G. salinus* increased by an order of magnitude during the laboratory experiment, in the case of *G. locusta*, there was either no change between the number of individuals at the beginning and that at the end of the experiment, or the number decreased resulting in the negative growth of the population (Table 1; Figure 3). At the end of the laboratory experiment, the mean and standard deviation of *G. locusta* individuals in the control, one heatwave and three heatwaves treatment were 30.3 ± 17.8, 27.8 ± 10.6 and 18.0 ± 8.5, respectively, while those of *G. salinus* was 137.0 ± 44.9, 152.3 ± 88.3 and 114.8 ± 89.5, respectively. Finally, neither the one heatwave nor three heatwaves treatment demonstrated any significant change in the percentage of population growth for either of the species in either experimental type (ANOVA, $F(2,18) = 0.838$, $p = 0.448$ and ANOVA, $F(2,18) = 0.392$, $p = 0.681$ for the mesocosm and laboratory experiment, respectively).

	Control				One Heatwave				Three Heatwaves			
Gammarus locusta												
Mesocosm experiment												
Beginning	48	48	48	48	48	48	48	48	48	48	48	48
End	559	247	371	620	493	616	666	883	1039	210	413	580
R	1065	515	773	1292	927	1183	1288	1740	2065	338	760	1108
Laboratory experiment												
Beginning	20	20	20	20	20	20	20	20	20	20	20	20
End	27	56	15	23	20	27	43	21	20	20	6	26
R	35	180	-25	15	0	35	115	5	0	0	-70	30
Gammarus salinus												
Mesocosm experiment												
Beginning	48	48	48	48	48	48	48	48	48	48	48	48
End	373	668	159	144	538	309	566	295	444	290	615	456
R	677	1292	231	200	1021	544	1079	515	825	504	1181	850
Laboratory experiment												
Beginning	20	20	20	20	20	20	20	20	20	20	20	20
End	152	80	187	129	140	65	275	129	89	127	229	14
R	660	300	835	545	600	225	1275	545	345	535	1045	-30

Table 1 Number of individuals in each replicate for both species (*Gammarus locusta* and *G. salinus*) at the beginning and at the end of both experiments (i.e. mesocosm and laboratory) in all three treatments (i.e. control, one heatwave and three heatwaves), and the respective population growth (*R*).

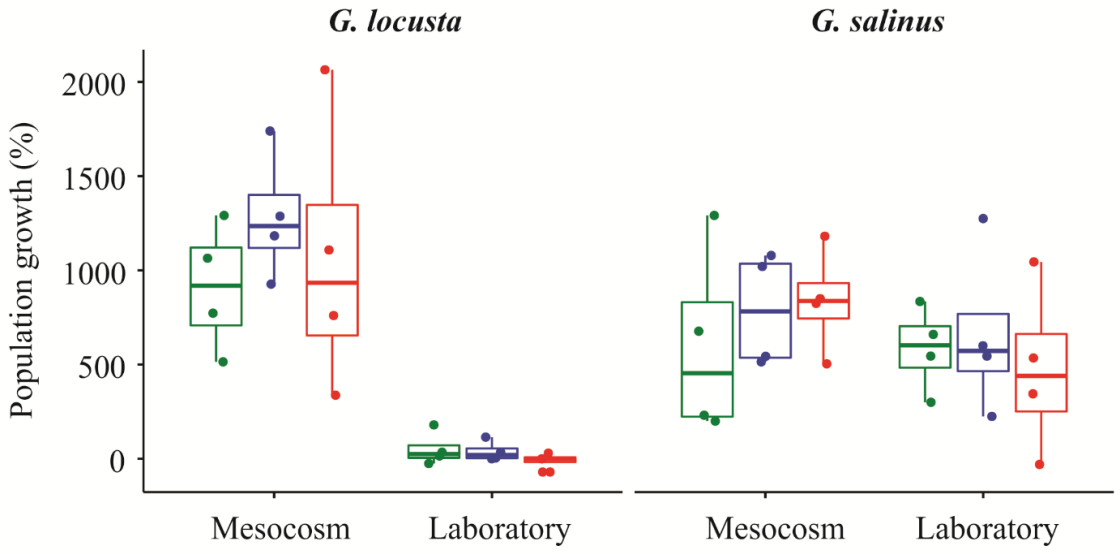

Fig. 3. Population growth (%) of individuals of *Gammarus locusta* and *G. salinus* in both mesocosm and laboratory experiments. Experimental treatments are depicted in green, blue and red for control, one heatwave and three heatwaves, respectively. The boxes represent upper and lower quartiles with median line, while dots represent the separate replicates.

Discussion

While laboratory experiments are mostly focused on specific physiological and ecological responses to environmental changes of individuals or a single species, mesocosms embrace a higher complexity by including assemblages of a population subset which increases the possibility of biological interactions and 'ecosystem realism' (Stewart et al., 2013; Widdicombe et al. 2010). However, there is still considerable uncertainty with regard to both types of experiments, and to which extent their results can be extrapolated and generalized with confidence. Our comparative assessment of mesocosm with laboratory experiment revealed that while for one species the results were similar independently of the experimental type, for the other species, the larger area of the mesocosm accompanied with inclusion of the community benefited the species' growth rate, demonstrating stronger performance in the mesocosm than in the laboratory experiment. Though, we acknowledge here that our study design did not allow to distinguish if the scale or ecological

complexity of the experiments, or both, caused the observed discrepancy between the two types of experiments. However, at the same time, our results revealed no difference in the heatwave impacts on any of the tested species independently of which experimental type was used.

The potential impacts of climate change on coastal marine environments and freshwater ecosystems have been extensively studied since the early 1990's where most studies have mainly focused on the species level (Harley et al., 2006; Wrona et al., 2006 and references therein). Lately, much more information using mesocosm experiments became available, which has been used, in some way, to calibrate and confirm that laboratory experiments properly represent natural ecosystems and their interactions (Schindler, 1998). Interestingly, our results have led us to two different conclusions for the two studied species that were tested using different experimental types. Independently of the heatwave treatment, *G. salinus* results were similar regardless of the experimental type, while for *G. locusta*, our study revealed differences between the two experimental types, with much poorer performance of individuals under laboratory conditions. Therefore, if one would study competition between these two species under current and/or future global warming scenarios under laboratory conditions, they could conclude that *G. salinus* would outcompete, or reduce population size, of *G. locusta*. Considering that settings in our study were exactly the same for both species in both experimental types, we believe that *G. locusta* is more sensitive to laboratory setting than *G. salinus*. Yet, our study design does not differentiate if the scale or ecological complexity of the experiments, or both, were responsible for the observed results. Under low salinity environments, several laboratory studies, for instance Bulnheim (1979) and Paiva et al. (2018), have determined that *G. locusta* is the most sensitive species among the Baltic gammarids with the lowest capacity to survive. In addition, this species reveals a much higher oxygen intake when exposed to such stress in comparison to other species, which seems to

explain its absence from polluted areas (Costa & Costa, 2000; Bulnheim, 1979). Another explanation could be that the small space of the tanks used in the laboratory experiment in addition to the absence of natural predators and food availability, have triggered possible fighting and cannibalistic behavior within and among species. Our laboratory experiment has been started by two orders of magnitude higher density of each species than those in the mesocosm experiment. Therefore, density-dependent effects experienced in the laboratory conditions may have been different from those in the mesocosms, causing bias in the observed results. As reported by Dick (1995), *Gammarus* spp. are omnivores with tendency to strong predatory activities, especially on susceptible individuals such as juveniles, and might prey on congeneric in order to get nutritional profits. Though cannibalism occurs under natural and semi-natural conditions, decreased structural habitat complexity might increase predatory activity (Christie & Kraufvelin, 2004; Dick, 1995; Dick & Platvoet, 1996; Macneil & Prenter, 2000). In addition, cannibalism and intraguild predation have been suggested as regulation factors when population density is too high (Christie & Kraufvelin, 2004). Consequently, though artificial refuges were provided in our laboratory experiment, these might have been ineffective due to the high density of individuals and consequent density-dependent effects since the two studied species are potential competitors for both space and food. As at the end of the experiment, we observed the majority of individuals of *G. locusta* being adults, as well as unchanged abundance of *G. locusta* between the beginning and the end of the experiment, we believe that the predation on its juveniles occurred, affecting the population growth rate of the species. Finally, due to unpredictable behavior of species in highly artificial environments, such as laboratory experiments, we emphasize a necessity of great caution when testing and interpreting results on species interactions and/or impact on each other.

While the overall aim of experiments is to provide essential knowledge of current and future threats to diverse communities or to study species and/or communities in general, the conducted experiments may not always be representative of natural systems (Cooke et al., 2017; Kraufvelin et al., 2006, 2010, 2020; Widdicombe et al., 2010). In fact, our study raised an important question considering the reliability of our own laboratory experiment and laboratory types of experiments in general. In the mesocosms experiment, half of the tested species demonstrated tolerance to heatwaves, which included both *G. locusta* and *G. salinus*, with only few species responding strongly negatively (see Pansch et al., 2018). However, in apparent contrast to the observed lack of sensitivity to heatwaves of *G. locusta* in both experimental types in our study where during the last heatwave temperature reached 25.2°C, previous laboratory studies have found high mortality rate of this species above 20°C and 22°C and suggested future global warming scenario exceeding the thermal limit of the species (Cardoso et al., 2018; Neuparth, Costa & Costa, 2002). Similarly, *Marenzelleria viridis*, a successful invader in Baltic waters, revealed a positive effect of heatwave treatments on both its biomass and abundance in our mesocosm experiment (see Pansch et al., 2018). However, Bochert, Fritzsche & Burckhardt (1996), using laboratory experiments, found a temperature of 20°C to be too high for a proper development of the species suggesting an abnormal growth during larval phase. Therefore, while the laboratory experiment of our study produced similar results to the mesocosm experiment when testing tolerance of species to heatwaves, our experiments were contrasting findings of previously conducted laboratory experiments testing resilience of those species using constantly elevated temperatures (Bochert et al., 1996; Cardoso et al., 2018; Neuparth et al., 2002; Pansch et al., 2018). As the results of laboratory experiments are not being consistent, and sometimes contradict the results of mesocosm experiments, we stress a need of conducting more comparative studies

between mesocosm and laboratory experiments to determine to which extent they are comparable and represent reality in nature. Though, as a result of this study, we trust that use of mesocosm instead of laboratory experiment is advocated whenever possible, as available space and/or community interactions are of paramount importance for species fitness.

Though some organisms may, or may not, show resistance in controlled laboratory experiments when exposed to a single, or multiple stressors, their sensitivity may change when exposed to other factors, such as the complex physical components and biotic interactions of the natural environments (Sommer, Adrian, Bauer & Winder, 2012). Although such laboratory experiments may improve our knowledge on the physiological response of the individuals, they are not a true replication of what occurs in nature. Actually, our study strongly indicated that the same species may respond differently when tested at high density and isolated from a community than when at lower density and in the presence of a subset of a community, confirming recent finding by Wahl et al. (2020). Furthermore, inconsistency in results among laboratory experiments complicates the extrapolations and generalization of the laboratory results even more. Our findings indicate the importance of scale, density, biotic interactions and complexity of natural environments in buffering, or boosting, the direct effects of environmental stress on organisms. Therefore, we urge the use of mesocosm experiments whenever possible, and emphasize a necessity of great carefulness when interpreting and generalizing the results of laboratory experiments.

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Chapter 5

General Discussion

Geographic origin and invasion success

The impacts of human activities are among the most critical pressures on biodiversity, contributing to shifts in the distribution of native species and facilitating the establishment and range extension of invasive species (Capinha et al., 2015; Halpern et al., 2008). In addition, the translocation of species has increased worldwide, some incidents of which have been facilitated by climate warming, reaching areas previously protected by natural barriers such as retreating sea ice in the Arctic (Chan et al., 2018). In the last decades, invasion ecology has expanded to several fields of research including improved prevention, by using mathematical modeling to help us to understand and predict the behavior of complex systems, and on the molecular level, using novel genetic approaches to understand possible traces of invading populations (Canning-Clode, 2015). However, the fact that biological invasions are still happening every day makes it clear that there are possibly unknown characteristics, mechanisms and processes that require further investigation, that might influence invasion success. The present thesis focusses on the North and Baltic Seas, the Ponto-Caspian and the North American regions. While these systems are far apart geographically and have distinct geological evolutions, more than a century of continuous species introductions facilitated by strong heavy shipping traffic contributed to a very similar biodiversity composition (Reid & Orlova, 2002; Ricciardi &

MacIssac, 2000). However, differences in the magnitude of species present among these systems, with Ponto-Caspian taxa prevailing in both Northern European and in the North American regions, suggested that Ponto-Caspian species would be better invaders than species from other regions (Casties et al., 2016; Reid & Orlova, 2002). Moreover, the much higher number of species from the Ponto-Caspian region observed in the Great Lakes - St. Lawrence River in comparison to the expected numbers calculated using shipping routes, available species pool from donor regions and environmental similarity, raised more questions concerning the influence of geographic origin on species invasion success (Casties et al., 2016). In this context, Chapter 2 of this thesis investigated whether Ponto-Caspian taxa more readily acclimatize to and colonize diverse salinity habitats than taxa from Northern European and North American regions. In these experiments it was found that although species from all three tested regions demonstrated a high tolerance to a wide range of salinity, significant differences in direction of salinity tolerance were observed among the regions, with Northern European species performing better in higher and Ponto-Caspian in lower salinities. These results suggest that euryhaline species from the Ponto-Caspian region might be of evolutionary freshwater origin, thus supporting previous studies (Casties et al., 2016; Reid & Orlova, 2002). In fact, discovering that species from this region in the Baltic Sea mostly settled in lagoons, estuaries and lower courses of rivers (Mordukhay-Boltovskoy, 1964; Zenkevitch, 1963) as opposed to more saline habitats such as the Black Sea or the Mediterranean Sea (Paavola, 2005; Shiganova, 2010) highly supports this theory. Interestingly, though species from the same region revealed the same salinity pattern, differences were found among populations of the same species. Furthermore, in both Chapters 2 and 3 the same populations were used when considering

Ponto-Caspian and Northern European species. However, it was only possible to confirm the salinity tolerance pattern seen in the experiments in Chapter 2 for juveniles (see Chapter 3). While as stated in Chapter 3, the use of pre-formed pairs in precopula was necessary to impose reproduction, single individuals were used in the previous experiments (see Chapter 2). The precopula stage can result in a number of energetic costs for both males and females (Elwood & Dick, 1990; Jormalainen, 1998; Sparkes, 1996), which of course might have had an influence on the animals' performance when exposed to extra environmental stresses such as salinity.

Life history stages

In the experiments conducted in Chapter 3, the three species were able to reproduce in different salinities. However, all juveniles experienced mortality in the course of the experiments, thus revealing a narrower tolerance than their parents. In addition, juveniles reproduced in fresh water conditions by Ponto-Caspian adults did not survive. Such results can be explained by the life history *r*-strategy that evolved among marine invertebrates, where high offspring production increases the chances of some individuals surviving into adulthood in the dynamic oceanic environment (Ramirez-Llodra, 2002). Also, since environmental stress might influence the availability of finite resources to organisms in order to maximize fitness, energy must be allocated to several different daily processes such as physiological maintenance and survival. Consequently, it is likely that reproduction was affected, resulting in weaker offspring (Mills & Fish, 1980; Neuparth et al., 2002; Steele & Steele, 1991). Nevertheless, differences observed in the hatching success among species were most likely related to their different reproductive strategies. Surprisingly, the only species tested without a known invasion record (Northern European *Gammarus*

salinus) was the only species capable of reproducing in very high and very low salinities. Yet, growth of juveniles in stressed treatments was rather low in comparison to the controls which once again suggests a possible allocation of energy to survival instead of growth.

The case of North American species

Considering North American species, conclusions about salinity tolerance of the tested species were inconsistent due to their poor performance under laboratory conditions. While control treatments often had high mortalities, this was not the case for the stressed treatments. The same pattern was also observed by Casties et al. (2018) who examined temperature tolerance. The reason for this rather contradictory result in the control group might be due to the presence of dark spots on tested animals, suggesting an infection by a parasite. This theory was further strengthened in the findings in Chapter 3, where juveniles had the lowest mortality rate in the control treatment compared to those in the low and high salinity treatments, and also compared to their parents and adults in any treatment tested in the previous experiments (see Chapter 2). It is possible that the parasite did not cope with environmental changes such as salinity and temperature, giving an advantage to those animals facing stress by making the impact of parasitism ineffective, which is known as the enemy release hypothesis in invasion ecology (Torchin et al., 2003). Parasitic oomycetes can reduce the immune function of animals and were already identified as a major cause of amphipod mortality in the St. Lawrence River, where the affected tested species are native (Kestrup et al., 2011). It is very likely that the transmission of such parasites does not occur from parents to offspring since no spots were found on juveniles and their survival was much higher than that of their parents in the control group. Thus, an additional parasite (e.g., microphallid trematodes) that needs an additional host might be

responsible for reducing the immune system of adults but does not affect juveniles (MacNeil et al., 2003; Mouritsen et al., 2005; Prugnolle et al., 2005). Nevertheless, the gammarid *Gammarus tigrinus* tested here is considered to be one of the major invasive species in the Baltic Sea, causing devastating impacts on native populations by outcompeting them (Jänes et al., 2015; Kotta et al., 2011). While the invasion success of this species is attributed to its high environmental tolerance (e.g., Sareyka et al., 2011; Wijnhoven et al., 2003), the same conclusion could not be observed in any of the experiments of any of the different populations.

Mesocosm and laboratory experiments - the importance of community

Chapter 4 of this thesis aimed at estimating possible influences of the spatial and complexity scales of experiments when results are obtained under strictly controlled conditions. In this context, two findings emerged among the tested species: i) the first species, *G. locusta*, demonstrated stronger performance in the mesocosms than in the laboratory experiment, and ii) for the second species, *G. salinus*, similar results were found among the two types of experiments. Such results could be explained by a higher sensitivity of *G. locusta* towards changing environmental conditions in comparison to *G. salinus* since settings were equal for both species. *Gammarus locusta* has previously demonstrated higher oxygen intake when exposed to salinity stress in comparison to other species, which seems to explain its absence from polluted areas (Bulnheim, 1979; Costa & Costa, 2000). Therefore, the presence of natural biological interactions in the mesocosm experiment, such as the provision of cooler micro-habitats or more oxygen supply around macrophytes, may have minimized such a susceptible state of the species. A recent study found that while ecological complexity buffered (and even reversed) the negative impacts of the elevated

presence of CO_2 on marine consumers, a strong negative impact on multiple behaviors were found in simpler experiments (Goldenberg et al., 2017). It is possible that while single individuals possess high plasticity to persist through abiotic stress of their environment, their interaction with other species can proliferate or stabilize during change (Goldenberg et al., 2017). This can lead to stronger and more complex indirect effects. In turn, the diversity of species improves not only the function but also the stability within ecosystems.

Conclusion and future directions

In conclusion, even though the tested species came from similar ambient salinities, differences were found in the direction of salinity tolerance, with Northern European species performing better in higher salinities and Ponto-Caspian ones in lower salinities. Such relevant results should be incorporated into predictive models. Ships undergo water exchange prior to arrival at a coastal or inland port to reduce the transfer of potentially invasive organisms by increasing the salinity, which can be fatal to some organisms (Briski et al. 2010; Molina & Drake, 2016). However, in some cases, such an exchange could act as a filter instead because of incomplete exchange, which consequently creates brackish environmental conditions and might be an advantage for certain species (Reid & Orlova, 2002). Thus, the colonization success of Ponto-Caspian species might be correlated with the fact that areas with greatest introduction frequency of NIS, such as shipping ports, are environmentally fluctuating habitats, which generally include freshwater inputs. Therefore, Ponto-Caspian species might not be innately better colonizers, rather, their intrinsic advantage may simply rely that such habitats are intolerable to euryhaline marine taxa. Furthermore, we must consider these results when predicting future impacts of climate change in order to anticipate future invasions by Ponto-Caspian species. According to

Meier et al. (2012), by the end of the twenty-first century, along with an increase in water temperature, a decline in salinity is anticipated for the Baltic Sea. Therefore, although Ponto-Caspian NIS are currently not established in higher salinity areas of the North and Baltic Seas (Casties et al., 2016; Paavola et al., 2005), it is expected that Ponto-Caspian species will spread further in the system, as well as in other areas with future salinity declines. Moreover, this doctoral thesis emphasized the need for studies testing populations from both native and non-indigenous regions, the results of which could provide valuable information on determining stress tolerance of diverse taxa. Additional research is required to confirm whether these findings can be generalized for species with an adequate ecological information on current invasion history, since more successful NIS are often more resistant to multiple stressors and pre-adapted to anthropogenic impacts (Holopainen et al., 2016; Hufbauer et al., 2011). By comparing salinity tolerance of adults and juveniles originating from Northern Europe, the Ponto-Caspian and North American regions, it was determined that juveniles were not able to tolerate the same stress as adults. This highlights not only the importance of considering the geographic origin of the species to predict changes in species distributions, but also how different the performance between adults and juveniles can be when exposed to stress. Nevertheless, additional studies are needed to confirm whether these findings can be generalized to other taxa. Therefore, different life history stages, which are often overlooked when testing the tolerance of species, should be considered when constructing models and when predicting resilience of ecosystems and biodiversity change. Finally, understanding both the cause and effect of coastal problems requires a diversity of research approaches, i.e., a broad spectrum of scales. Changes in the experimental design demonstrated that the same species may respond differently when

tested isolated from its community than in the presence of a subset of a community. In the context of invasion ecology, this might also help to understand why some species, presumably evolutionarily naïve to their new surroundings, can become invasive, but also how some might have the capacity to replace locally adapted natives. According to the community assembly theory, it is likely that the composition and relative abundance of species within a community are determined by a series of hierarchical filters that allow or impede the passage of each potential non-indigenous organism based on its functional traits (Pearson et al., 2018). Therefore, though small-scale laboratory experiments are important to improve our knowledge on physiological and some ecological responses, each individual experiment must be seen as a means of answering a specific question or providing specific knowledge that will advance our holistic understanding of the issue concerned. Taken together, we learn that not only geographic origin and life history stages need to be considered in invasion ecology, but also the approach when selecting our experimental designs to answer research questions. More experiments, including natural assemblages and communities (higher ecological complexity), are therefore vital to cover a range of groups, species, populations, functional groups and physiological traits as well as interdisciplinary fields (e.g., climate change and invasion ecology). The current research on biological invasions lacks information on the role of ecological complexity in potentially altering or stabilizing local communities during invasions. By recognizing the importance of the interconnection between species invasion and community invasiveness, we could investigate why some invaders become serious problems in one community but fail to do so in others.

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Contribution of authors

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Chapter 2:

Title: "Is salinity an obstacle for biological invasions?"

Authors: Filipa Paiva (FP), Andrea Barco (AB), Yiyong Chen (YC), Alireza Mirzajani (AM), Farrah T. Chan (FC), Velda Lauringson (VL), Miguel Baltazar-Soares (MBS), Aibin Zhan (AZ), Sarah A. Bailey (SB), Jamileh Javidpour (JJ), and Elizabeta Briski (EB)

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Contributions: EB designed the study, FP, AB, AM, FTC, VL, SAB and JJ collected the data, YC, MBS and AZ conducted the genetic analyses, EB processed the data, EB and FP drafted the manuscript, and all authors revised the manuscript.

Chapter 3:

Title: "Are juveniles as tolerant to salinity stress as adults? A case study of Northern European, Ponto-Caspian and North American species"

Authors: Filipa Paiva (FP), Nora-Charlotte Pauli (N-CP) and Elizabeta Briski (EB)

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Contributions: EB, FP and N-CP designed the study, FP and N-CP collected the data, FP and EB processed the data, EB and FP drafted the manuscript and all authors revised the manuscript.

Chapter 4:

Title: "Consistency of aquatic enclosed experiments: the importance of scale and ecological complexity"

Authors: Filipa Paiva (FP), Dennis Brennecke (DB), Christian Pansch (CP) and Elizabeta Briski (EB)

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Contributions: FP, CP and EB designed the experiments, FP, DB, CP and EB conducted the experiments and collected the data, FP and EB analyzed the data, FP and EB wrote the manuscript. All authors revised the manuscript and approved the last version of the manuscript.

Declaration

I, Filipa Paiva, hereby declare that the dissertation submitted, entitled “The role of geographic origin and life history stages in invasion ecology - a comparative assessment of Northern European, Ponto-Caspian and North American species” was written independently by me and only using the sources listed. The content and design of this thesis, apart from the supervisor’s guidance, is my own work. Chapter 2 and 3, of this doctoral thesis, were published in peer reviewed journals in 2018 and 2020, respectively, while Chapter 4 has been submitted to a peer reviewed journal and is currently under review. The contributions to the manuscripts made by myself and my co-authors are explained in the section “Contributions of authors”. This work has been prepared respecting the Rules of Good Scientific Practice of The German Research Foundation. I have not been deprived of an academic degree.

Kiel, 13.11.2020

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Filipa Paiva